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Maryland Univ., Baltimore. School of Medicine

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DETECTION OF MUTAGENIC/CARCINOGENIC ALTERATION IN FISH

By

David E. Hinton
James E. Klaunig
Michael M. Lipsky
Rhona M. Jack and
Benjamin F. Trump

Department of Pathology, University of Maryland
School of Medicine, Baltimore, Maryland 21201

Project Number R-804866-D1-0

Project Officers

Dr. Gary Glass and
Mr. James Tucker

Environmental Research Laboratory - Duluth
U.S. Environmental Protection Agency
Duluth, Minnesota 55804

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16. ABSTRACT

The feasibility of using fish as bioassay organisms to detect mutagenic/carcinogenic substances in the aquatic environment was tested. The data in fish were compared to those in higher vertebrates including man. Microsomal fractions from livers of channel catfish, fathead minnow, bluegill sunfish, brown bullhead, rainbow trout, and mummichogs were studied to determine the presence and relative amounts/activities of microsomal protein, cytochromes P-450 and b_5, NADPH cytochrome C reductase, aminopyrine demethylase, and aryl hydrocarbon hydroxylase. The effects, both morphologic (upon the endoplasmic reticulum) and biochemical, of exposure to known MFOS inducing agents, PCBs, benzo(a)pyrene, and 3-methylcholanthrene were studied. Exposure caused induction of enzymes and proliferation of endoplasmic reticulum membranes in hepatocytes. High pressure liquid chromatographic analysis of benzo(a)pyrene (BP) metabolism in catfish and trout was performed. Both species produced 9,10-diol BP; 4,5-diol BP; 7,8-diol BP; 9-OH BP; 3-OH BP; and quinones. Catfish postmitochondrial supernatant converted BP and AAF into mutagenic intermediates in a microbial mutagen system. Catfish liver cells were isolated and maintained with high viability (98%) for 10 days. When incubated with ^{3}H-BP, these cells showed preferential accumulation of label over nuclei. Subsequent liquid scintillation analysis of cell fractions obtained by cesium chloride centrifugation revealed radioactivity in DNA fractions. Foci of hepatocellular alteration, hyperplastic areas and bile ductular hyperplasia were seen in channel catfish chronically exposed to AAF. Granulomatous lesions occurred in livers of mummichogs chronically exposed to FBPA. These indicate the suitability of conducting further studies on these species designed to determine dose response characteristics to various chemical carcinogens. On the basis of microsomal metabolism and cellular response, it appears feasible to use fish tissues to test for mutagenic/carcinogenic compounds in the aquatic environment and to develop bioassay methodology for testing possible carcinogenic properties of new chemical formulations prior to their introduction ito the aquatic environment.

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PREFACE

It should be emphasized that this was a feasibility study and as such was not designed to "mimic" environmental conditions but rather to determine whether certain events in chemical carcinogenesis, previously determined to be important in mammalian studies, would occur in specific fish species. In addition, two of the chemical carcinogens, N-(4'-fluoro-4-biphenylyl)acetamide (FBPA) and 2-acetylaminofluorene (AAF), are not known as environmental pollutants. These compounds are research carcinogens which have been studied in detail in rodent systems. Thus, they permit comparison of findings in fish with previous studies in rodents. The latter two compounds, benzo(a) pyrene and 3-methylcholanthrene (which have also been studied in detail in rodent systems) are implicated in human carcinogenesis and are relevant environmental pollutants.

ABSTRACT

The feasibility of using fish as bioassay organisms to detect mutagenic/carcinogenic substances in the aquatic environment was tested. The data in fish were compared to those in higher vertebrates including man. Microsomal fractions from livers of channel catfish, fathead minnow, bluegill sunfish, brown bullhead, rainbow trout, and mummichogs were studied to determine the presence and relative amounts/activities of microsomal protein, cytochromes P-450 and b_5, NADPH cytochrome C reductase, aminopyrine demethylase, and aryl hydrocarbon hydroxylase. The effects, both morphologic (upon the endoplasmic reticulum) and biochemical, of exposure to the known MFOS inducing agents, PCBs, benzo(a)pyrene, and 3-methylcholanthrene were studied. Exposure caused induction of enzymes and proliferation of endoplasmic reticulum membranes in hepatocytes. High pressure liquid chromatographic analysis of benzo(a)pyrene (BP) metabolism in catfish and trout was performed. Both species produced 9,10-diol BP; 4,5-diol BP; 7,8-diol BP; 9-OH BP; 3-OH BP; and quinones. Catfish postmitochondrial supernatant converted BP and AAF into mutagenic intermediates in a microbial mutagen system. Catfish liver cells were isolated and maintained with high viability (98%) for 10 days. When incubated with ^{3}H-BP, these cells showed preferential accumulation of label over nuclei. Subsequent liquid scintillation analysis of cell fractions obtained by cesium chloride centrifugation revealed radioactivity in DNA fractions. Foci of hepatocellular alteration, hyperplastic areas and bile ductular hyperplasia were seen in channel catfish chronically exposed to AAF. Granulomatous lesions occurred in livers of mummichogs chronically exposed to FBPA. These indicate the suitability of conducting further studies on these species designed to determine dose response characteristics to various chemical carcinogens. On the basis of microsomal metabolism and cellular response, it appears feasible to use fish tissues to test for mutagenic/carcinogenic compounds in the aquatic environment and to develop bioassay methodology for testing possible carcinogenic properties of new chemical formulations prior to their introduction into the aquatic environment.

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SECTION II

CONCLUSIONS

1. All fish species were found to contain a hepatic mixed-function oxidative system (MFOS) qualitatively similar to that of other teleosts and rodents.

2. The enzyme activity of the fish MFOS was increased by exposure to polychlorinated biphenyls and polycyclic aromatic hydrocarbons, benzo(a)pyrene (BP) and 3-methylcholanthrene (3-MC).

3. Increased enzyme activity correlated with proliferation of endoplasmic reticulum of hepatocytes.

4. Channel catfish and rainbow trout liver microsomal fractions metabolized BP into diols, hydroxy derivatives, and quinones.

5. Pretreatment of catfish and trout with 3-MC increased formation of 7,8- and 9,10-diols of BP. Since diol formation proceeds via epoxide formation, evidence indicates fish MFOS activated BP to a reactive carcinogen.

6. Catfish MFOS formed mutagenic intermediates of BP and 2-AAF in a microbial mutagen system.

7. *In vitro* organ explant cultures of fish kidney and skin can be maintained for up to 8 weeks and permit interspecies comparison of effects relative to carcinogenesis.

8. Isolated primary hepatocytes of channel catfish can be maintained with high viability for 10 days and permit detailed analysis of cellular events in chemical carcinogenesis as well as direct interspecies comparison.

9. The presence of foci and areas of hepatocellular alteration, nodular lesions and bile ductular hyperplasia suggest neoplastic responses to chronic carcinogen exposure in fish liver.

10. It is feasible to use fish to test for the presence of mutagenic/carcinogenic substances in the aquatic environment and to determine whether new chemical formulations, proposed for widespread usage, would have mutagenic/carcinogenic potential in aquatic species.

11. The chronic nature of laboratory exposures designed to demonstrate carcinogenicity of compounds results in sizeable quantities of contaminated water which require decontamination prior to discharge. The volume of contaminated water can be diminished by use of closed system exposure aquaria but remains a problem to be considered in projects of this nature.

SECTION III

INTRODUCTION

The purpose of this project was to determine the feasibility of using fish-derived cell fractions and cell and organ explant cultures in *in vitro* tests to detect mutagenic and carcinogenic potentials of substances; and, secondly, to determine whether tumors could be produced in certain teleost species not commonly employed in carcinogen bioassay.

According to World Health Organization estimates (1), over 75% of human cancers are influenced by environmental factors. There is now growing awareness that the majority of human cancers are due to chemical carcinogens in the environment (2,3). The importance of the aquatic environment in considerations of carcinogenic effects of chemical pollutants cannot be overly emphasized since it has been shown that the oceans ultimately become the reservoir for every pollutant entering the biosphere (4). From the standpoint of human health, the consumption of contaminated fish flesh (5) and contaminated drinking water (6,7) pose real and potential hazards. Biologic effects of aquatic pollutants related to neoplasia was the subject of a recent international symposium in which the need for suitable experimental models for studying carcinogens in water was emphasized (8).

Since fish reside in polluted waters and since analysis of feral bony and cartilaginous fishes for tumors has revealed neoplasms within virtually all organ systems (9-11) the possibility exists for use of fish as bioassay organisms in aquatic carcinogen research. Although the field of experimental carcinogenesis in aquatic organisms is in its infancy (12), some laboratory exposures of various fish species to established chemical carcinogens have resulted in tumor formation. TABLE 1 is a summary of data from laboratory tumor induction studies with fish. More detail may be found in recent reviews (11,13).

From TABLE 1 it is readily apparent that the liver is by far the most common site for primary tumors in laboratory exposures with various fish species. The epitheliomas seen after benzo(a)pyrene and methylcholanthrene were induced by direct application of compounds to the skin (24). Only two compounds, dimethylnitrosamine and nitrosomorpholine, produced primary tumors in other organs as well as liver (14,16). Since tumors occur in feral fishes and since they can be produced in the laboratory, it should be possible to (1) identify cancer frequencies, by monitoring fish in their native habitat, and (2) to test for carcinogenic potential of new products by laboratory exposure of suitable fish species. Global epizootic studies (25,26) have established frequencies

TABLE 1. TUMOR INDUCTION IN FISH BY CHEMICAL CARCINOGENS

CARCINOGEN	SPECIES	TUMOR & REFERENCE
Acetylaminofluorene	A	Hepatocholangiocarcinoma (14,15)
	B	Hepatocarcinoma (16)
Aflatoxins	A,B,C,D	Hepatoma (15,17-19)
Aminoazotoluene	A	Hepatocholangiocarcinoma (14)
	B	Hepatocarcinoma (16)
Cycad nut meal	E	Neoplasms of liver (20)
Methylazoxymethanol	F	Neoplasms of liver (21)
DDT	B	Hepatocarcinoma (16)
Diethylnitrosamine	A	Hepatocholangiocarcinoma (14)
	E,F	Hepatocarcinoma (22,23)
Dimethylaminoazobenzene	A,B	Hepatocarcinoma (14,16)
Dimethylnitrosamine	A	Hepatocholangiocarcinoma (14,15)
	B	Hepatocarcinoma & Nephroblastoma (16)
Nitrosomorpholine	A,E	Hepatocholangiocarcinoma & intestinal adenocarcinoma (14)
PAH	G	Epithelioma of skin (24)
Benzo(a)pyrene	G,H	Epithelioma of skin (24)
Methylcholanthrene	G	Epithelioma of skin (24)
Urethane	B	Hepatocarcinoma (16)

A - *Lebistes reticulatus*.
B - *Salmo gairdneri*.
C - *Oncorhynchus nerka*.
D - *Salvelinus fontinalis*.
E - *Brachydanio rerio*.
F - *Oryzias latipes*.
G - *Gasterosteus aculetus*.
H - *Rhodeus amarus*.

of skin papillomas in marine flatfish and eel populations. Such data on tumor prevalence, when related to water quality and to laboratory-determined causative agents, may serve as an "early warning system" for carcinogens in the aquatic environment.

However, as Stich and Acton (27) have indicated, the ultimate purpose of the "early warning system" would be to indicate potential hazards to human health. As with any animal model system, the comparison of results in experimental animals with those obtained from the human population remains one of the really great unsolved problems in cancer research (27). Thus, the sensitivity and types of responses of fish to chemical carcinogens, is a baseline issue that must be investigated in detail prior to comparison with other animal models in man. Furthermore, appropriate test systems amenable to interspecies comparison are needed.

Background information on chemical carcinogenesis.

Most chemical carcinogens are not immediately reactive; they require metabolic activation. For many procarcinogens, this activation is achieved by drug-metabolizing enzymes in the mixed-function oxidative system (MFOS). The final product is a chemically reactive form, the ultimate carcinogen (28,29). The MFOS is located predominantly in the microsomal fraction of cells. This fraction, when studied by electron microscopy contains endoplasmic reticulum, Golgi apparatus and plasma membranes (30). The mammalian MFOS is a multicomponent electron transport system, the enzymes of which are bound to the membranes of the endoplasmic reticulum (ER). Substrate is activated by a monooxygenase system which is a group of nonspecific drug metabolizing enzymes unique in their versatility and requiring NADPH and molecular oxygen for activity. Constituents include two protein components; cytochrome P-450, a heme protein, and NADPH cytochrome P-450 reductase, a flavoprotein (31).

Cytochrome P-450, so-called because it binds carbon monoxide in its reduced form resulting in a characteristic absorption spectrum with a maximum at 450 nm, is important because of its role in oxygen activation, substrate binding, and determining the overall substrate specificity of the enzyme system (31). NADPH cytochrome P-450 reductase is an electron carrier necessary for the transfer of electrons from NADPH to cytochrome P-450 during microsomal hydroxylation (32). It contains one molecule each of FAD and FMN per polypeptide (33) and also reduces other electron acceptors such as cytochrome c, which is one basis of its measurement. One NADPH-cytochrome c reductase molecule reduces several cytochrome P-450 molecules (34).

The lipid component, phosphatidylcholine, is believed necessary for electron transport from NADPH-cytochrome c reductase to cytochrome P-450; however, the lipid microenvironment may be a requirement for activity (35). Another hemoprotein, cytochrome b_5 may play a part in the electron transport as well. The extent of b_5 involvement may depend on the substrate and form of cytochrome P-450 involved, since it has been shown that there are numerous forms of cytochrome P-450 (36).

The following steps from Gillette et al. (31) are common to most oxidative reactions catalyzed by the MFOS.

1. Substrate combines with oxidized cytochrome P-450 to form a complex.

2. The complex is reduced by an electron from the flavoprotein NADPH-cytochrome c reductase. This step may be rate limiting since maximal rates of ethylmorphine demethylation by liver microsomes from various species are more closely related to the rate of reduction of the cytochrome P-450-substrate complex than they are to the amount of cytochrome P-450. Similarly, differences in the rate of cytochrome P-450 reduction account for the differences in the rate of ethylmorphine demethylation between smooth and rough surfaced microsomes from rabbit liver (37).

3. The reduced complex reacts with oxygen, forming a substrate-cytochrome P-450-oxygen complex.

4. A second electron, possibly donated by NADPH or NADH or mediated by cytochrome b_5, reduces the complex, giving rise to an active oxygen intermediate and H_2O.

5. Decomposition of the intermediate occurs with the formation of products and oxidized cytochrome P-450.

The lipid fraction, phosphatidylcholine, causes an increase in reduction of cytochrome P-450 even though the lipid does not contain components that undergo redox reactions (33). Without phosphatidylcholine, drug metabolism is inhibited. The fluidity of the phospholipid is also essential for interaction between cytochrome P-450 and NADPH-cytochrome c reductase. The microsomal enzymes interact with phospholipid membranes, and activity is dependent on membrane integrity (35,36).

The general function of the MFOS is metabolism of endogenous compounds such as steroids, fatty acids, and bile acids, as well as exogenous compounds (xenobiotics) such as carcinogenic polycyclic aromatic hydrocarbons (PAH) including benzo(a)pyrene (BP) and 3-methylcholanthrene (3-MC), drugs (phenobarbital), insectides, and other industrial compounds such as polychlorinated biphenyl (PCB) and polybrominated biphenyls (PBB) (31,37,38). It has been well-documented (39-41) in rodent liver studies that the administration of a drug such as phenobarbital, or a PAH causes SER proliferation, an increase in the metabolism of the drug, and an increase in the specific activity of hydroxylating enzymes. Eventually resistance to drugs such as phenobarbital is built (42).

Induced enzyme synthesis begins in the rough endoplasmic reticulum (RER) and leads eventually to accumulation of smooth endoplasmic reticulum (SER), rich in hydroxylating enzymes (36). The synthesis of a new membrane is tied to formation of new enzymes. The mechanism may involve an outgrowth and budding off of SER from RER at sites where enzyme synthesis takes place. Membrane proliferation is also accompanied by an increase in phospholipid synthesis and a slowing down of catabolism (36) as has been shown in rat liver following PCB exposure (43). The induction process also leads to an increase in levels of both flavoprotein and cytochrome components of the MFOS. Also involved are increases in rates of protein synthesis and RNA synthesis as well as a decrease in the rate of breakdown of the flavoprotein. All these factors contribute to an increase in enzyme content.

PAH, an important class of environmental pollutants which cause tumors in experimental animals including fish (24,44,45) and have been implicated in human carcinogenesis (46), are activated by the NADPH-dependent MFOS (47,48). That portion of the MFOS responsible for this metabolic activation is the aryl hydrocarbon hydroxylase (AAH) system (47).

In the case of one PAH, BP, the result of activation by AAH is formation of an epoxide, which is further modified by an epoxide hydrase to form a dihydrodiol. A simplified interpretation of the metabolic scheme of BP as modified from Selkirk (49) is outlined below (see Fig. 1). The epoxides may also non-enzymatically open to form hydroxy derivatives. Glutathione transferases catalyze the conjugation of intermediates to form water soluble (excretable) substances. There is also some metabolism of oxides back to the parent PAH which is NADPH-depedent and inhibited by oxygen (50).

HPLC is, therefore, an essential tool with which to clarify the complexities of BP metabolism. By HPLC, examination of species differences is possible and comparison of data from fish, rodent and man may be made.

It is generally true that metabolically activated forms of carcinogens (i.e., proximate or ultimate carcinogens) are electrophilic and as such seek out negatively charged regions in target molecules and form strong covalent chemical bonds (53). Such covalent binding between the carcinogen metabolite and cellular macromolecules, such as DNA, can lead to initiation of the carcinogenic process (46). Carcinogen binding is tested in in vitro organ explant and tissue culture systems. Isotopically labeled carcinogen is incubated with tissue in culture and autoradiograms are used to demonstrate location of label (nucleus or cytoplasm). Radioactivity per macromolecule (DNA, RNA, protein) is used to quantify the response. These tests have been employed in rodent and human studies (54,55).

Binding to DNA, an integral step in the carcinogenic process for genotoxic carcinogens, may result in altered expression of genetic material (i.e., mutation). This led to an association between carcinogenicity and mutagenicity. This correlation was tested by Ames et al. (56) in a bacterial mutagenesis system and has formed the basis of a widely used screening procedure (57). When known carcinogens were tested, 90% were found to produce mutations by this procedure (58).

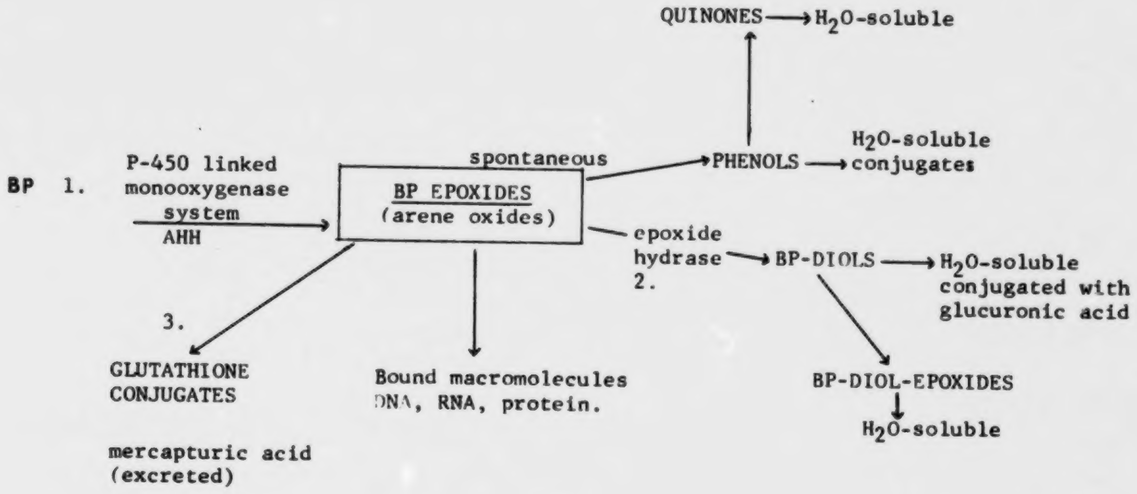
QUINONES → H_2O-soluble
P-450 linked monooxygenase system AHH
BP 1.
spontaneous
BP EPOXIDES (arene oxides)
PHENOLS → H_2O-soluble conjugates
epoxide hydrase 2.
BP-DIOLS → H_2O-soluble conjugated with glucuronic acid
3.
GLUTATHIONE CONJUGATES
mercapturic acid (excreted)
Bound macromolecules DNA, RNA, protein.
BP-DIOL-EPOXIDES
H_2O-soluble

Figure 1. Metabolism of benzo(a)pyrene.

In measuring microsomal monooxygenase enzyme induction, one common approach is to assay AAH activity. This method gives an overall view of increased or decreased activity of the enzyme system, but does not give a clue as to how the substance is being metabolized. Recently (51), high pressure liquid chromatography (HPLC) has been perfected to elucidate the metabolites of benzo(a)pyrene. Metabolite patterns of benzo(a)pyrene are tissue specific, with a great deal of difference between species (49). Furthermore, prior induction of the MFOS may specifically alter pathways of metabolism relevant to carcinogenesis (52). Pretreatment of male rats with 3-MC increased the specific activities of liver microsomal enzymes toward the formation of all BP metabolites. However, induction did not have a uniform effect on the formation of each metabolite. Using HPLC, Yang and associates (52) saw an increased specific activity toward formation of benzo(a)pyrene 9,10 dihydrodiol (9,10 diol BP) and benzo(a)pyrene 7,8 dihydrodiol (7,8 diol BP).

Several exceptions to the correlation between mutagenicity and carcinogenicity exist (59). DDT and other chlorinated hydrocarbons, hormones such as diethylstilbestrol and naturally occurring estrogens as well as drugs (phenobarbital) caused tumors in animal bioassays yet were uniformly inactive in mutagenicity tests. In addition, some carcinogens exert their effects through incompletely understood mechanisms that might best be called epigenetic interactions (59).

Due to these differences in mechanisms, carcinogens can be classified into two broad types, genotoxic and epigenetic. Because no single test has detected all the known genotoxic carcinogens, multiple *in vitro* tests are necessary.

The *in vitro* tests have specific advantages in screening of potentially carcinogenic compounds. Some of these include: ease of handling, economy of operation, ability to make interspecies comparisons, and greatly decreased time between initiation of studies and final data accumulation. However, at the present, there exists no substitute for whole body, lifetime exposures to establish by the presence or absence of malignant neoplasms whether a substance is in fact a carcinogen. The prior use of *in vitro* tests may yield indications as to suitable species for long term bioassay and meaningful estimates for concentraton of the toxicant to be tested. Estimates from 1977 (53) show a cost of $300,000.00 per compound for complete carcinogenicity testing in rodent species. Since hundreds of compounds need to be tested, the use of alternate species which may prove more economical needs to be investigated. In this regard, the short latency period for tumor development in small aquarium fish, the resistance to the toxic effects of certain test carcinogens, and the decreased animal maintenance costs when compared to rodent systems (13) were strong indicators that additional studies to ascertain responses of fish to known carcinogens were needed. Thus, this feasibility study was initiated to study discrete steps in the carcinogenic process in fish species not heretofore tested.

Initial studies with fish suggested an absence of a MFOS. It was assumed that fish could dialyze lipid-soluble substances through their gill membranes (60). The elasmobranch gill can, in fact, dispose of only 1-2% of a given dose of a common MFOS substrate (61).

However, as in mammalian systems, a MFOS capable of functioning in the normal oxidative metabolism of bile acids, fatty acids, and steroids exists in fishes. Testosterone metabolism was studied in the hagfish, a primitive vertebrate, and in teleosts (62). It was concluded that teleosts contain similar steroid reductive enzymes to those found in mammals. The hydroxylation reactions which allow for bile synthesis are also common to fish and mammals (61,62).

Since 1962, more detailed studies describing oxygen and NADPH-requiring hepatic enzymes have been reported in fish liver (61,63-67). As in the mammalian system, carbon monoxide and cytochrome c inhibit skate hepatic MFOS, while cytochrome P-450, NADPH-cytochrome c reductase and lipid fractions are all required for maximal activity (67). AHH has been demonstrated in livers of both freshwater (66,68) and marine species (64,67,69-71).

MFOS activity in fish shows variation with respect to species (64, 71), strain (72), size (73), sex in gonadally mature individuals (74) and chemical treatment or environment (67,69,75).

Several studies have examined induction of the MFOS in fish with PAH and with polychlorinated biphenyls (PCBs). Induction, as measured by increased AHH activity, has been demonstrated in trout with as little as 4 mg 3-MC/kg b.w. (72). Gerhart and Carlson (76) found 300 ug BP/kg b.w. to be the minimum effective dose for induction of cytochrome P-450, AHH and aniline hydroxylase in rainbow trout. Commercial mixtures of PCBs, when administered to fish caused induction of ethoxycoumarin-o-deethylase (77), cytochrome P-450 (78-80), cytochrome b_5 and NADPH-cytochrome c reductase (79,80), aniline hydroxylase (81) and aminopyrine demethylase (79-81).

Until recently, the sensitivity of fish MFOS to PAH had not been fully appreciated. Two administrations of 20 mg 3-MC/kg b.w. caused 8-11 fold AHH increases in a marine teleost species (69). Statham et al. (81) exposed rainbow trout to various PAHs and found dramatic increases in AHH concomitant with no effect on glucuronyl transferase. Using thin layer chromatography, Ahokas et al. (83) showed that trout liver microsomes converted BP to dihydrodiols and hydroxy metabolites at a rate 5-10 times higher than rat liver microsomes. When activity was expressed per unit cytochrome P-450 or NADPH-cytochrome c reductase, the rate of metabolism was 15-30 times faster than in rats. Stegeman and Chevion (74) demonstrated higher levels of AHH in fish than in rodent liver when AHH was normalized to cytochrome P-450 amounts in each species.

At the initiation date of this project, data were available which demonstrated the presence of a microsomal mixed function oxidative system in certain marine (64) and freshwater fish species (66,83-85). Functional studies had demonstrated the capacity for metabolism of drugs (63), insecticides (65, 86), chemical carcinogens such as 2-AAF (87) and benzo(a)pyrene (83) and induction by polychlorinated biphenyls (78). In addition, metabolism of PAH had been demonstrated in cell cultures isolated from rainbow trout and bluegill sunfish (88). No high pressure liquid chromatographic (HPLC) analysis of BP metabolites had been performed using fish derived cell fractions; and, similarly no application of fish-derived tissue fraction had been reported in conjunction with bacterial mutagenesis studies. As is noted in TABLE 1, laboratory exposure of fishes to chemical carcinogens resulted primarily in liver tumors. However, the species employed in those studies were almost entirely restricted to salmonids and small aquarium fishes. If one or more ubiquitous species, sensitive to various carcinogenic agents, could be found, fish models for environmental carcinogen studies could be established. In addition, laboratory trials with new products, destined for widespread usage, which may become environmental pollutants, could be performed using these systems to screen for potentially carcinogenic activity. Thus, this project was undertaken to ascertain the responses of fish species, not heretofore used in carcinogen bioassay, to proven laboratory carcinogens.

In order to achieve our overall objective, certain enabling objectives, divided into the following four phases, were pursued:

1. Characterization of liver MFOS in various species and its response to known inducing compounds;

2. Application of bacterial mutagenesis assays using fish liver post mitochondrial supernatant;

3. Development of suitable fish tissue and organ explant culture systems and utilization of same in in vitro studies with labeled BP; and

4. Long-term continuous exposure to known carcinogenic compounds for the purpose of inducing neoplastic lesions.

SECTION IV

MATERIALS AND METHODS

General Procedures

Animals: Fish

Fish for experimentation were obtained from the following sources: Private hatchery - channel catfish (*Ictalurus punctatus*), fathead minnow (*Pimephales promelas*), bluegill sunfish (*Lepomis macrochirus*) and brown bullhead (*Ictalurus nebulosus*); Department of Natural Resources, State of West Virginia - trout (*Salmo gairdneri*); trapped from the Chesapeake Bay at Oxford, Maryland and kindly donated to us by personnel of the National Marine Fisheries Service Laboratory - mummichogs (*Fundulus heteroclitus*).

All fish were acclimated for a period of at least 2 weeks in 100 gallon Living Stream aquaria (Frigid Units, Inc.) and observed for bacterial and parasitic infections prior to use in experiments. During this period, all fish were fed trout chow (Purina). Fish food was not analyzed for organic contaminants. Laboratory holding rooms were maintained with 12 hour alternating light and dark photoperiods.

Channel catfish, fathead minnows, bluegill sunfish and brown bullheads were maintained in charcoal filtered water from the city utility at 20°C. Trout were held similarly but at 12°C. *Fundulus heteroclitus* were maintained in 20 gallon glass aquaria at a salinity of 20 parts per thousand (Instant Ocean, Aquarium Systems, Inc.) at 20°C. Water quality was monitored weekly and maintained at 6.8-7.2 pH, 8-9 mg/liter dissolved O_2 and less than 1 part per million ammonia nitrogen concentration. As closely as possible, the specifications established by the Committee on Methods for Toxicity Tests with Aquatic Organisms (89) were followed.

Rats

In order to standardize fish assays and to compare enzyme data in fish with rats, male Sprague-Dawley animals 125-175 g were used. These were maintained in stainless steel cages with wire mesh bottom and fed laboratory chow (Purina). Food and water were available *ad libitum*.

Preparation of Microsomal Fractions

Some fish were killed by severing the spinal cord while others were anesthetized by submersion in a 1:4000 solution of tricaine methane sulfonate (MS-222) (90). In order to remove blood from livers, anesthetized fish were perfused with a Ringer's solution using a whole body perfusion technique (90). Fish were subsequently dissected and livers were rapidly removed, blotted, weighed and placed in appropriate solutions (0.5M Tris - 1.15% KCL or 0.3M sucrose, pH 7.4) on ice. In order to remove blood from non-perfused livers, minced pieces were rinsed repeatedly in Tris-KCL and placed in fresh buffer. A 25% homogenate was made by four complete passes in a glass Potter Elvehjem tube with a teflon pestle at 260 rpm. Unbroken cells, nuclei, mitochondria and lysosomes were removed by centrifugation at 10,000 x g for 15 min. In order to obtain a microsomal pellet, the resulting supernatant was sedimented at 100,000 x g for 60 min. The pellet was resuspended in either 0.3M sucrose or 0.5M Tris-1.15% KCL buffer to a final concentration of 0.5 grams liver/ml microsomal suspension. Centrifugations were carried out in refrigerated Beckman or Sorvall ultracentrifuges at 0-4°C. For characterization studies, "pooled" microsomal fractions from four control fish were used. Microsomal preparations were frozen and stored at -80°C until time of assay. Experimental analysis of storage techniques revealed no loss of activity under these conditions. Microsomal protein concentration was determined by the method of Lowry et al. (92).

Microsomal Enzyme Assays

The cytochromes P-450 and b_5 were determined according to the method of Omura and Sato (93). The amounts were estimated using extinction coefficiencts of 91 nM^{-1} cm^{-1} between 490 and 450 nM (p-450) and 170 nM^{-1} cm^{-1} between 424 and 409 (b_5).

Since hemoglobin in tissues can interfere with the quantitation of the cytochromes (P-450 and b_5) we used whole body perfusion via aortic cannulation with saline to remove blood. This technique (91) is suitable for larger fish; however, small fish were difficult and often impossible to perfuse. In order to use smaller fish, we developed a perfusion procedure, using micromanipulators and a suction device with needle to deliver saline. Success rates of perfusion by this method exceeded 80%.

The device is pictured (Figure 2).

Glass tubing, sealed over a 30 guage needle (Becton-Dickson) was extended to the level of the bevel of the needle. A hole was drilled in the side of the glass tube and polyethylene tubing was sealed over the hole. At the opposite end, the tubing was connected to the lumen of rubber surgical tubing by an air-tight seal. The 2" piece of surgical tubing was sealed by plugs at each end. Small, anesthetized fish were placed in a trough the floor of which contained moist gauze and their pericardial cavities were opened via dissection through the ventral body wall over the heart. The ventricular wall was isolated and by using a microscope stage, the needle and glass tubing were advanced until a snug fit against the ventricular wall was obtained. At this time the 2" piece of surgical tubing was depressed and released creating a vacuum which pulled the ventricular myocardium over the needle tip. In this way, the needle tip was introduced into the ventricular cavity and flow was initiated at a rate of 130 μ liters/min. Perfusion fluid forced blood from the circulation via the cut vessels of the body wall. This procedure was used to remove blood from livers and to deliver electron microscopic fixative solutions.

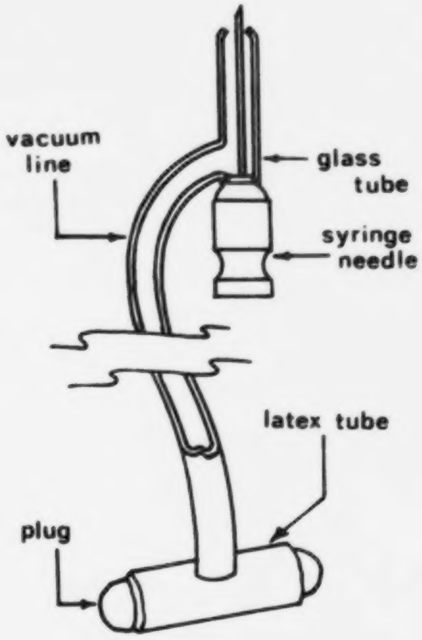

Figure 2. Apparatus for perfusion of small fish.

Aminopyrine demethylase was determined by the method of Gnosspelius et al. (94). The amount of formaldehyde formed was determined using the Nash reagent (95). The activity of NADPH cytochrome-c-reductase was determined by the method of Dallner et al. (96). An extinction coefficient of 19.1 nM^{-1} cm^{-1} at 550 nm was used to estimate the specific activity. Aryl hydrocarbon hydroxylase (AHH) was determined by the fluorometric method of Nebert and Gelboin (97). All protein estimates followed the method of Lowry et al. (92).

AHH Assay

AHH activity was measured by the fluorometric method modified from Nebert and Gelboin (97) using a Farrand ratio fluorometer and Corning filter no. 5-58 as the excitation filter (400-460nm), and filter no. 3-70 as the emission filter (wavelengths above 500 nm). The assay volume of 1 ml contained 0.1M phosphate buffer (pH 7.2), 3.0 mM MgCl , 0.1 mM EDTA, 10mM glucose-6-phosphate, 0.4 mM NADP, 1.5 Units of glucose-6-phosphate dehydrogenase, and 0.1-0.2 mg microsomal protein. Protein concentration within each characterization study was kept constant.

Reactions were initiated by the addition of 100 nmoles of substrate (BP) in 25 ul acetone. Tissue blanks received either 1 ml of acetone prior to the addition of BP to inactivate the enzyme, or microsomes were incubated in 25 ul acetone without BP. No difference was found between fluorescence of the two blanks. The assay was carried out in a shaking water bath at 30°C and terminated at 20 min by addition of 1 ml cold acetone. Metabolites were extracted into 3.5 ml hexane. Subsequently, a 2 ml aliquot of the upper hexane phase was vortexed with 1.2 ml 1N NaOH added to extract phenolic products of BP. The fluorescence was measured after determining the fluorescent equivalent for the formation of 1 nmole of 3-OH-BP with authentic standard. Specific activity was calculated as nmoles 3-OH-BP released/20 min/mg protein.

In the 7,8-benzoflavone (BF) inhibition studies, BF was added in 20 ul acetone. Samples not getting inhibitor received 20 ul acetone. These studies were done to see whether BF inhibition would distinguish different forms of cytochrome P-450 in untreated and treated fish.

Chemicals - AHH Assay

Glucose-6-phosphate dehydrogenase was obtained from Boehringer Mannheim Corp., Indianapolis, IN. BP and 3-OH-BP standard were obtained from IIT Research Institute, Chicago, IL. All other materials were from Sigma Chemical Co., St. Louis, MO.

Light Microscopic Techniques

Tissues for light microscopy were fixed in 4% formaldehyde-1% glutaraldehyde (4F1G) in 200 milliosmolar phosphate buffer at pH 7.4. This fixative has been shown to be of value for light and electron microscopic study (98). Hematoxylin and eosin (H&E) stains were used for routine survey of all tissues. Periodic acid/Schiff's reagent (PAS) stain was used for survey and for analysis of connective tissue. For visualization of lipids in tissues, an oil red O stain of frozen fixed material was used.

Electron Microscopic Techniques

Tissue for transmission electron microscopy was fixed as above but included fixation by whole body perfusion (91) and by immersion. Since the 4F1G fixative was used on all tissues, stored tissues are also available for subsequent electron microscopic work-up as required. Using a clean razor blade, slices of the outer 1-2mm portions of organs were made and placed in pools of fixative, minced into 1mm pieces, postfixed in OsO_4, dehydrated in graded ethanol solutions and embedded in Epon (99). For correlation between light and electron microscopy and to better visualize hepatic lobular regions, semithin sections (0.5-1 u) were cut on an ultramicrotome, stained with toluidine blue (100) and viewed with a light microscope. In addition, the increased resolution made possible by the decrease in section thickness permitted the study of rearrangement of organelles such as is seen with proliferation of smooth endoplasmic reticulum. Thin sections (600-1000A) were mounted on copper grids, stained with uranyl magnesium acetate and lead citrate and viewed under an electron microscope.

Tissues for scanning electron microscopy were fixed as above, dehydrated in graded concentrations of ethanol, critical point dried with CO_2, coated with gold palladium and viewed under an AMR-1000 scanning electron microscope (101).

HPLC Methods

The procedure for HPLC analysis was modeled after Selkirk et al. (51). Metabolites were formed by the reactions of fish liver microsomal fractions with 80-100 nmoles of tritiated substrate (BP) with a specific activity of 150-200 uCi/umole. Each ml of assay volume included 0.1 M phosphate buffer, pH 7.0-7.2, 3.0 mM MgCl , 0.1 mM EDTA, 0.4 mM NADP, 10 mM glucose-6-phospate, 15-20 uCi BP, and 1.5 Units of glucose-6-phosphate dehydrogenase as the NADPH generating system. BF (0.1mM) was added in 20 ul of acetone in some experiments. Protein concentration was maintained at 0.1 mg per assay. After incubation in a shaking water bath at 30°C for 20 min, the reaction was stopped by the addition of 1ml acetone. The contents of 10 assay tubes were combined, and metabolites were extracted 3 times with ethyl acetate. Radioactivity remaining in the aqueous phase was less than 0.1% of the total. The ethyl acetate extracts were pooled, flash evaporated, and the metabolites were resuspended in 0.1 ml of glass distilled methanol.

Metabolite separation was performed using a Varian high pressure liquid chromatograph with a 25 cm Whatman Partisil column (PXS10/250DS) of inside diameter, 4.6 mm. Column temperature was ambient. For analysis, 10-20 ul of the methanolic extract was injected into the HPLC.

A methanol-water gradient was used to elute metabolites. Two solvents designated `a` and `b` were used. Solvent `a` was 30:70 methanol: water, and solvent `b` was 70:30 methanol:water. Initially, concentration of `b` in `a` was 25%. This was increased linearly at 3%/min and at a flow rate of 1.2 ml/min to 100% `b`. This reverse phase chromatography, proceeding from a more to less polar solvent, elutes polar components first. Approximately 200 fractions were collected (0.4 min/ fraction) in order to elute all metabolites and unmetabolized parent BP which, due to its nonpolar nature, is eluted last.

All samples were co-chromatographed with ^{14}C-labeled BP metabolites from rat liver microsomes as an internal standard or with pure individual metabolites to validate identification of peaks (absorbance monitored at 254 nm). Since the rat liver BP metabolite pattern is well characterized, it is often used as a standard. Peaks were also identified by retention times established with pure metabolite standards.

Radioactivity of eluted fractions was determined by adding 4 ml Aquasol, followed by counting in a Packard liquid scintillation counter (model #3385). The ^3H counts were computed to obtain specific activity for each metabolite, and expressed as pmoles BP metabolized/min/mg protein. The ^{14}C counts were used only for identification purposes. The sum of counts from fractions collected prior to the final BP peak represented total BP metabolized. Individual metabolites were expressed as a percent of this total. In this manner, quantitative differences in activity of various metabolites were compared in control and treated fish to establish effect of in vivo exposure of xenobiotics upon in vitro BP metabolism.

Chemicals

^3H-labeled BP was obtained from Amersham (Arlington Heights, IL), and ^{14}C labeled BP was obtained from New England Nuclear (Boston, MA). Unlabeled BP was obtained from IIT Research Institute (Chicago, IL), as were the pure BP metabolite standards. Glucose-6-phosphate dehydrogenase was from Boehringer Mannheim Corp (Indianapolis, IN), and other reagents were from Sigma Chemical Co. (St. Louis, MO). Glass distilled ethyl acetate and methanol were from Burdick and Jackson (Muskegon, MI). Aquasol was obtained from New England Nuclear (Boston, MA).

Organ Explant Culture Techniques

Fish for culture work were removed from holding aquaria and placed in a 20 gallon glass tank containing sterilized, charcoal-filtered Baltimore City water and were maintained in this water for at least 24 hours before transfer to the tissue culture area. Immobilization of fish was achieved by severing the spinal cord. Skin was the first tissue removed. An area along the lateral body was washed with sterile saline. Following this, multiple parallel cuts, 0.5 cm deep x 1.0 cm wide, were made along the entire length of the fish. The skin was then removed in strips by grasping one end with rat-tooth forceps and cutting with a new sterile scalpel blade. Gills were removed by cutting dorsal and ventral arch connections. Viscera were dissected from the body after the outer surface was wiped clean with 70% ethanol. An incision along the ventral midline was made from the pectoral girdle to the anal opening. Liver, kidney, intestine and swim bladder were removed intact. Following removal, all tissues were immediately placed in petri dishes containing cold (4°C) Leibowitz (L-15) medium. Prior to subsequent culture, gill, skin, liver and kidney pieces were trimmed into smaller units to facilitate media diffusion. Intestine was split longitudinally to expose luminal surface. Swim bladder was cut into 1 cm pieces.

Trimmed tissues were placed into 60 mm sterile plastic petri dishes. Skin, swim bladder, and intestine were oriented with epithelium uppermost. CMRL medium (GIBCO) containing per ml: 1.0 ug of hydrocortisone hemisuccinate (Schwartz/Mann, Rockville, MD), 1 ug of bovine recrystallized insulin (Schwartz/Mann), 300 units of penicillin, 300 ug streptomycin (GIBCO) was used. Culture dishes were placed in a controlled atmosphere chamber (Bellco Glass Co., Vineland, NJ) and gassed with 45% O_2, 50% N_2, 5% CO_2 for approximately 5 min. The chamber was then placed on a rocker platform which rocked at 10 cycles/min causing the media to flow intermittently over the epithelial surface. Explants were incubated at either 37°C or 25°C. Medium and atmosphere were replaced 3 times each week. These techniques were adaptations of Barrett et al. (101) which were developed for human organ explants.

Isolation and Culture of Fish Liver Cells

Under aseptic conditions, intact livers were removed, placed in sterile saline solution in petri dishes and minced into 1-2 mm pieces with sterile scissors. After additional rinses in saline solution to remove blood, pieces were weighed and placed in a 250 ml trypsinizing flask (Bellco). Liver cells were separated by continuous stirring in trypsin (0.25% in Ca^{++} and Mg^{++}-free Hanks' salt solution) until only a white fibrous plug remained (8 hrs at 25°C). The solution containing cells was filtered through gauze, to remove large clumps of cells and debris, spun at 200 x g for 5 min and the resulting pellet of liver cells was resuspended in Leibowitz's L-15 medium containing 10% fetal calf serum. Yield and viability (trypan blue exclusion) were determined by counting on a hemocytometer. For culture, 5×10^6 liver cells were plated onto each 25 cm^2 tissue culture flask (Falcon) with 5 ml of additional L-15 medium containing (per ml) 100 U penicillin, 100 ug streptomycin and 0.25 ug amphotericin. Media were changed daily.

Microbial mutagen assays - These followed methods of Ames and associates (56-58,103). *Salmonella typhimurium* tester strains TA-1535, TA-1537, TA-98, and TA-100 were obtained from Dr. Bruce Ames (University of California, Berkeley). Stock cultures were frozen and stored in liquid nitrogen. Working cultures were obtained by scraping the frozen permanents and inoculating scrapings in 25 ml Delong flasks containing 10 ml nutrient broth (trypticase soy yeast extract). After 16 hrs culture at 37°C, strains were plated (concentration of 1×10^6 cells/plate) onto 90 cm petric dishes, routinely checked for rfa (deep rough) character by crystal violet sensitivity and monitored for amphicillin resistance (strains TA-98 and TA-100). Supernatants (postmitochondrial), from a 9,000 x g spin (S-9) of liver homogenates were prepared as described by McCann et al. (57) and stored at -80°C until time of assay. Following solubilization in DMSO or isotonic saline solution, varying concentrations (0-30 ug) of test compounds AAF, BP, fluorobiphenyl acetamide (FBPA) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) were added to an NADPH generating system and to S-9 (10-150 ul in volume and from 0.167 to 2.94 mg protein concentration), mixed with top agar and poured onto preset Minimal Davis (Difco) plates with 2% glucose. In order to incubate NADPH generating system, S-9 and test compound directly on petri dishes with bacterial tester strains (57), an incubation temperature of 37°C was used. In previous experiments with catfish liver AHH, 70% of the activity at temperature optimum (30°C) was seen when assay temperature was maintained at 37°C. Following incubation, the number of revertant colonies was established by direct counting. For a biological control, S-9 from livers of rats previously exposed to Aroclor 1254 was used in companion trials with fish S-9 (103).

For autoradiographic localization of BP within isolated liver cells, primary cultures (1 x 10^6 cells) were plated onto 35 mm x 10 mm petri dishes containing a 25 mm^2 plastic coverslip (Thermanox, Lux Co.) and 2 ml L-15 medium. After 24 hrs H-BP dissolved in DMSO (final concentration 0.5%) was added to the culture medium to a final concentration of 1.25 uM (32 uCi/ml of medium). Cultures were incubated for 24 hrs at 25°C. After exposure, coverslips with attached liver cells were washed 3 times in L-15 to remove free BP. In order to visualize individual grains, nuclei were swollen by treatment with 1% sodium citrate. Cells were fixed in 3 changes (10 min each) of acetic acid-ethanol (1:3 v/v) and air dried. Cover slips were mounted (cells up) to clean glass slides, coated with emulsion (Kodak-NTB-2) and incubated at 4 C for two weeks. Autoradiographs were developed in Dektol and stained with hematoxylin and eosin. The number of grains over 100 nuclei and equally-sized but randomly-selected areas of adjacent cytoplasm and intercellular space (background) was determined and expressed as mean ± 1 standard deviation for each culture. In order to validate nuclear localization of the labeled BP, radioactivity of DNA isolated via differential centrifugation in cesium chloride (104) from 5 x 10^6 liver cells exposed to ^{3}H-BP was determined.

Test Chemicals

Aroclor 1254, an industrial mixture of various PCB's with impurities including TCDD was obtained (Monsanto, St. Louis, MI) and used in intraperitoneal injections to induce components of fish and rat MFOS. In addition, the following carcinogenic compounds were used in the study: 2-acetylaminofluorene (2-AAF); benzo(a)pyrene (BP); N-(4'-fluoro-4-biphenylyl)acetamide (FBPA); N-methyl-N'-nitro-N-nitrosoguanidine (MNNG); and 3-methylcholanthrene (3-MC).

Selection of the above compounds was made on the following basis. 2-AAF is a proven laboratory carcinogen in both mammalian (28) and fish systems (16) and is known to require microsomal activation (28). BP and 3-MC are examples of PAH's which are known environmental pollutants (105). These compounds have been implicated in human carcinogenesis (44) and have been shown to produce epitheliomas in the skin of fish (24). BP and 3-MC have been studied extensively as to their metabolism and are known to require microsomal metabolism to the ultimate carcinogenic metabolite. FBPA is a laboratory carcinogen which has been shown to cause tumors of the liver and kidney in rodents (106,107). This compound was chosen because of our extensive experience with it in producing tumors of the liver and kidney in the rat (108-110). MNNG is one of the two classes of compounds that does not require microsomal activation to the ultimate carcinogen (28) and was used in the Ames mutagenic bioassay as a positive control.

Diet Preparation

The above noted test compounds were dissolved in corn oil with sonication to ensure proper dispersal. Corn oil-carcinogen mixtures were then added to increasing amounts of trout chow (Purina) to reach the desired concentration. Mixing in a mechanical mixer of stainless steel construction was performed for one hour to ensure uniformity within the diet mixture. An equal volume of corn oil was added to trout chow (without carcinogen) and fed to controls. All diets were made monthly and stored in appropriately labeled sealed containers in a cold room (4°C) until one hour prior to feeding.

Safety Procedures

All laboratory personnel were instructed in the care and handling of toxic/carcinogenic substances and wastes. Entry to exposure rooms and to areas where bulk carcinogens were stored was restricted to appropriately trained personnel. Signs reading "Danger-potential cancer-causing agents used" were posted on all doors into exposure areas. Personnel were required to wear disposable gloves, masks and gowns when in exposure rooms. Bulk storage of carcinogens was in an appropriately labeled glove box and weighing plus initial dilution of compound in carrier oil took place within the glove box. In order to reduce aerosol formation, all exposure tanks were equipped with lids. In order to reduce volume of contaminated water, static exposure conditions were maintained. Although this method of maintaining fish undoubtedly led to increased mortality among exposed animals, it was selected to restrict safety hazard to personnel and reduce volume of contaminated water. Periodically aquarium water was replaced with fresh dechlorinated water. All waste water was filtered over charcoal beds prior to disposal. Used charcoal beds were incinerated.

Necropsy Procedures

Fish were killed by "pithing" and gross external and internal examination as made of each fish. In small specimens, examination was facilitated by use of a dissecting scope and 2-3 X magnification. Skin with underlying musculature, gills and viscera were examined and 2-3 mm slices were fixed by immersion in Bouin's or neutral buffered formalin solutions. In some instances, nodules or other suspect areas were placed in 4% formalin-1% glutaraldehyde and processed for Epon embedment and high resolution light microscopy. All other histologic procedures followed those already described.

SECTION V

EXPERIMENTAL PROCEDURES

In phase 1 of the study, the major objective was to determine the existence and characterize the response of certain MFOS components in channel catfish and to compare via correlated biochemical and morphologic techniques acute responses to PCB (Aroclor 1254 as 7 daily intraperitoneal injections of 50 mg PCB/kg b.w.) in livers of channel catfish and rat. Secondly, a similar approach was used with rainbow trout exposed to 3-MC (50 mg/kg i.p. x 4 days).

Next, studies were performed to characterize AHH from livers of control fish and from livers of fish exposed to 3-MC or to Aroclor 1254. Microsomal preparations were made as described in Section IV. AHH was characterized as to pH, temperature, and protein concentration optima and linearity of reaction over time was established.

In addition, determinations were made of components of the MFOS which could be induced by various agents including some carcinogenic compounds of environmental concern. Acute and subacute exposures via intraperitoneal (i.p.) injection, gastric insertion (g.i.) of a gelatin capsule, and dietary feeding were used.

Dose-response studies using channel catfish injected (i.p.) daily for four consecutive days with one of the following doses of 3-MC in corn oil: 10; 25; 50; or 100 mg/kg b.w., were performed. Three fish were used for each dose. Four control fish were injected with corn oil only. Fish were sacrificed 24 hrs following the last injection.

A duration of effect study was also performed. Twelve fish were injected (i.p.) on four consecutive days with 25 mg 3-MC/kg b.w. Three fish were killed at each time, at the following intervals: 1, 2, 4, or 7 days following exposure. The concentrations of 3-MC were chosen based on results from previous experiments in this laboratory. Injection volume was 0.5 ml, and care was taken to avoid leakage of injected material.

As a portion of Phase I, we performed HPLC studies of BP metabolism by microsomal fraction from livers of channel catfish and rainbow trout. These were performed as detailed in Section IV and included analysis of control, 3-MC and PCB treated fish. All peaks from fish were compared to pure standard metabolite patterns or to peaks after rat liver microsomal metabolism of ^{14}C-BP (biological control) (see Section IV).

In Phase 2, the major objective was to determine whether fish microsomal enzymes (in the S-9 fraction) could activate procarcinogens to mutation-producing compounds. To achieve this, the Ames mutagenic bioassay was set up as described in Section IV and appropriate fractions from rat and channel catfish were compared. Initially, we had planned to compare human S-9 with the animal fractions; however, human liver specimens were not available during the last year of the grant period. As recommended by Ames et al. (56), varying concentrations of test compounds and varying concentrations of S-9 were used.

In Phase 3, major emphasis was on the development of suitable fish organ explant and tissue culture systems for utilization in in vitro carcinogen studies. Although some cell culture systems were available and had been developed for fish, these were primarily fibroblasts or epithelial cells of ill-defined origin (88). Thus, it was necessary to develop culture explants of organs which had been shown to be involved in tumor development, namely liver, kidney, and intestine. In addition, other tissues were tried for organ explant culture. These are detailed in Section IV. Our attempts to maintain liver explant cultures were unsuccessful. Because the liver is a key target organ in experimental carcinogenesis of fish (see Section III), we elected to attempt to develop isolated liver cell cultures for in vitro carcinogen binding sites. Isolated liver cell cultures had been developed and played a key role in carcinogenesis studies in the rat (88).

The organ explant culture protocols were followed as established in Section IV and specimens were taken for morphologic work-up at varying times throughout 8 weeks in culture. The isolated liver cell cultures were analyzed morphologically up through 10 days in culture. These isolated liver cells were then exposed to ^{3}H-BP as described in Section IV.

Phase 4 Long-Term Exposure to Fish to Carcinogens

In order to determine the tumorigenic potential of the carcinogens tested in the in vitro experiments, chronic dietary administration of carcinogens to fish was initiated. Fish were fed diets containing FBPA, AAF, BP or 3-MC. All carcinogens were dissolved in corn oil and thoroughly mixed with Purina trout chow to the desired concentration (Table 2). Fish were fed daily at a level of 1% of the biomass. Channel catfish, mummichogs and Koi were held in living stream aquaria at constant temperatures (as described in Section IV). Brown bullheads and fathead minnows were maintained in 20 gallon glass aquaria equipped with undergravel filtration. Each experimental group was maintained in separate aquaria. The species, number of fish, compound and concentration used in the long-term protocols is given in TABLE 2. Following histologic staining and slide preparation, tissue sections were blind coded and each slide was read independently by three investigators. Findings reported represent a concensus of individual evaluations.

TABLE 2. CHRONIC CARCINOGEN EXPOSURE PROTOCOLS

SPECIES	# FISH	TREATMENT	DURATION OF EXPOSURE
Ictalurus punctatus	100	AAF (100 mg/kg diet)	14 months
	100	FBPA (.04% of diet)	14 months
	100	CONTROL	14 months
Ictalurus nebulosus	30	3-MC (100 mg/kg diet)	24 months
	30	BP (100 mg/kg diet)	24 months
	30	CONTROL	24 months
Pimephales promelas	50	3-MC (100 mg/kg diet)	24 months
	50	Control	
Fundulus heteroclitus	50	BP (100 mg/kg diet)	4 months*
	50	FBPA (.04% diet)	4 months*
	50	CONTROL	4 months*
Japanese imperial carp	50	FBPA (.04% diet)	9 months
	50	CONTROL	9 months

*Mechanical failure caused termination of exposure.

SECTION VI

RESULTS AND DISCUSSION

Hepatic Mixed-Function Oxidase System

As reviewed in the introduction to this report (Section III), it is now well-established that the hepatic MFOS is responsible for metabolism of xenobiotics and that continued exposure to many of these compounds results in increased amounts and activities of MFOS enzymes. In mammals, biochemical induction is accompanied, morphologically, by increases in the relative amounts of hepatocyte smooth endoplasmic reticulum (SER). Although effects of MFOS inducers, many of which are important environmental pollutants (i.e., PCB and BP), had been studied in fishes (see introduction Section I, this report), only Scarpelli et al. (111,112) had performed correlated biochemical and ultrastructural analysis in teleost liver. Those reports dealt with aflatoxin exposure in rainbow trout. Thus, studies in our laboratory were performed to characterize the normal MFOS of various teleosts and to study, via a correlated biochemical and morphologic approach, the effects of a few select inducers which are also carcinogenic.

Our results on hepatic MFOS components of the channel catfish and rainbow trout and their response to PCB and 3-MC exposure are summarized (TABLE 3). As a biologic control for comparison purposes, the response of the rat MFOS to acute PCB exposure is given (TABLE 4).

PCB

Acute PCB exposure (7 days) resulted in a moderate increase in some MFOS components. Cytochrome P-450 increased approximately 33% in channel catfish. Specific activity of NADPH-cyt-c-reductase was increased to 1.6 times control values. The greatest change was observed in amount of cytochrome b_5, which increased 2.5 fold. Aminopyrine demethylase activity, microsomal protein and liver to body weight ratios did not change after acute exposure. In contrast, rat MFOS components were markedly increased after an identical concentration and duration of PCB exposure (TABLE 4).

Subacute (21 days) of PCB treatment resulted in a large increase in catfish MFOS components with respect to controls and acutely exposed fish (TABLE 3). Amounts of cytochromes P-450 and b_5 increased 3 and 2 times over controls respectively. NADPH-cyt-c-reductase was elevated to a level similar to that seen after the acute exposure. Aminopyrine demethylase, unchanged by acute treatment, was increased 3 fold over controls after subacute treatment. Again, microsomal protein and liver to body weight ratios were unchanged. Thus, the response of the catfish MFOS to acute PCB treatment is a moderate induction, much less than is seen in the rat. Subacute exposure resulted in a greater degree of induction.

When compared to control (Fig. 3), hepatocytes of rats acutely exposed to PCB showed increases in cytoplasmic lipid and marked proliferation of SER as tubular profiles (Fig. 4).

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When compared to control (Figure 3), hepatocytes of rats acutely exposed to PCB showed increases in cytoplasmic lipid and marked proliferation of SER as tubular profiles (Figure 4).

TABLE 3. EFFECTS OF PCB ON CATFISH AND 3-MC ON TROUT HEPATIC MFOS

SPECIES	Cytochrome P-450^a	Cytochrome b_5^b	NADPH-cyt-c-reductase^c	Aminophyine demethylase^d	Microsomal protein^e	Liver/body wt. X10^f
Channel Catfish (Control)	.201±011	.237±0.13	19.15±6.1	1.7±0.53	11.20±3.6	1.3±0.4
Channel Catfish (PCB-acute)	.273±0.1*	.702±0.3(A)	26.72±5.8*	1.42±0.12	11.68±2.0	1.5±0.33
Channel Catfish (PCB-subacute)	.470±0.17(A)	.670±0.16	23.72±3.41*	5.98±1.86(A)	12.46±1.86	1.20±0.31
Rainbow Trout (Control)	.175±0.05	ND	11.24±2.47	ND	21.03±10.2	1.4±0.2
Rainbow Trout (3-MC)	.597±0.2(A)	ND	25.62±2.61*	ND	16.42±1.03	1.3±0.1

a) nmoles/mg protein; b) nmoles/mg protein; c) nmoles cyt-c-reduced/min/mg protein; d) nmoles HCHO/min/mg protein; e) mg/g liver.

* Significantly different with respect to control at $P < 0.1$.

(A) Significantly different with respect to control at $P < .05$.

All other values not significantly different from controls.

1 and 3a represent the mean ± 1 s.d. of 15 liver samples.

3b represents the mean ± 1 s.d. of 8 liver samples.

2 and 4 represent the mean ± s.d. of 3 pools of 4 liver each.

ND = not determined.

TABLE 4. EFFECTS OF ACUTE PCB EXPOSURE ON RAT HEPATIC MFOS

a. Cytochrome P-450	b. Cytochrome b_5	c. NADPH-cyt-c-reductase	d. Aminopyrine demethylase	e. Microsomal Protein	f. Liver wt.	
Control	.804±0.08	.50±0.13	121.±79.20	4.59±0.67	16.3±3.36	3.4
PCB	4.18±0.72	.579±0.11*	297.4±41	7.75±1.1	23.3±5.8	4.5

* Not significantly different from control. All other values significant at $p < 0.01$.

a) nmoles/mg protein.
b) nmoles/mg protein.
c) nmoles cyt-c-reduced/min/mg protein.
d) nmoles HCHO formed/min/mg protein.
e) mg/g liver

Control and PCB values represent the mean ± s.d. of 5 liver samples.

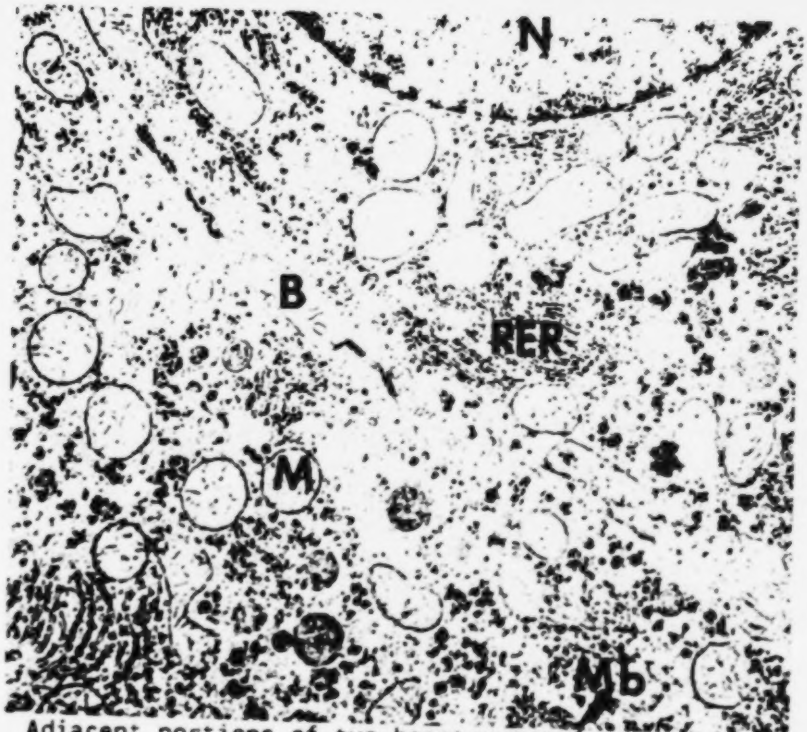

FIGURE 3. Adjacent portions of two hepatocytes from control rat liver show typical cytoplasmic features. RER, rough endoplasmic reticulum; M, mitochondrion; mb, microbody; B, bile canaliculus; N, nucleus. X 16,000.

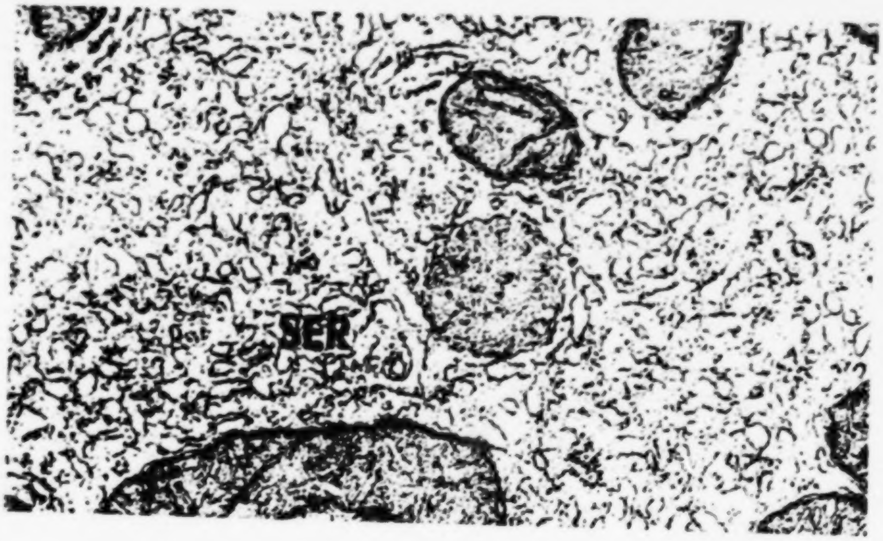

FIGURE 4. A portion of a rat hepatocyte following PCB exposure shows tubular profiles of smooth endoplastic reticulum (SER) which fill the cytoplasm. X 28,000.

Changes were also seen in hepatocyte ultrastructure of channel catfish after acute PCB exposure. When compared to control morphology (Figure 5), dilated cisternae and meandering tracts of ER were observed (Figure 6). Minimal increases in SER profiles were noted. These appeared as focal, discreet aggregation (Figure 7).

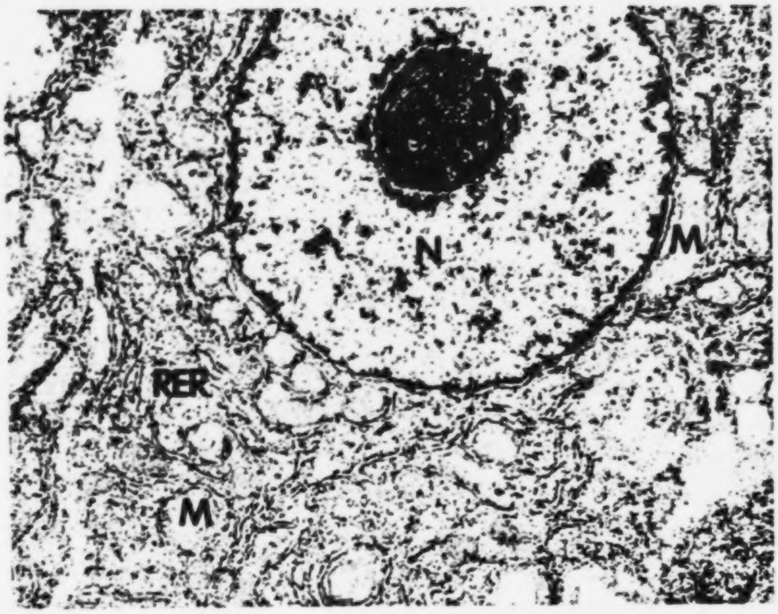

FIGURE 5. Electron micrograph from control fish liver showing normal appearance of nucleus (N) and cytoplasm. The cytoplasm contains mitochondria (M) and rough endoplasmic reticulum (RER) as parallel cisternae. X 18,000.

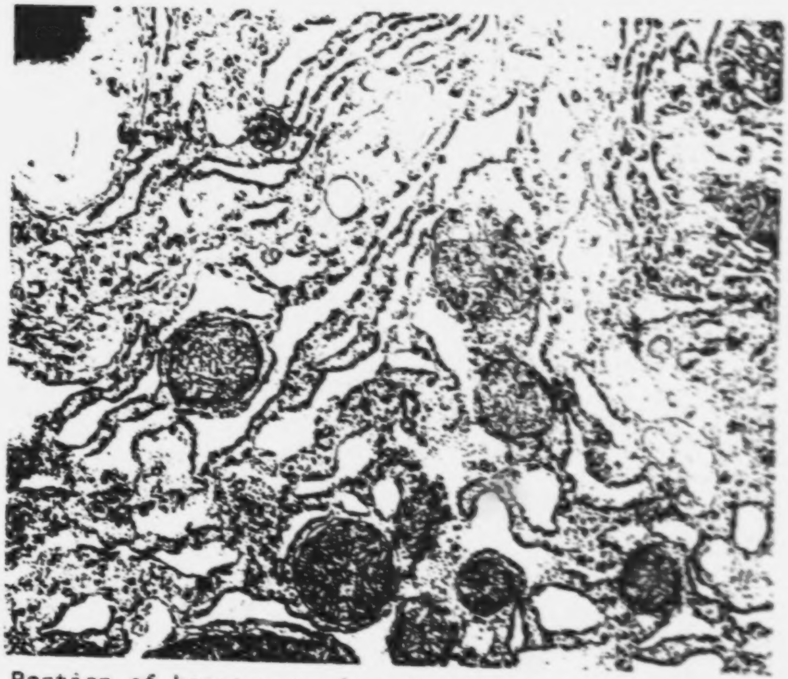

FIGURE 6. Portion of hepatocyte from PCB-exposed fish showing dilated cisternae of RER filled with material of low electron density. X 27,000.

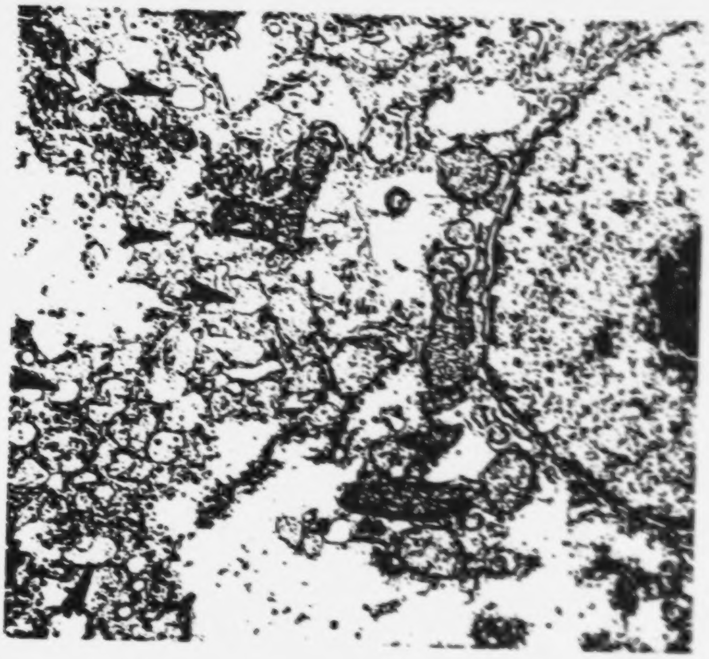

FIGURE 7. Hepatocyte from PCB-exposed fish shows focal accumulation of smooth surfaced membranes (arrows). X 20,000.

Increased lipid droplets, similar to those reported by Couch (112) in PCB-exposed marine teleosts, also characterized hepatocytes of acutely-exposed channel catfish (Figure 8).

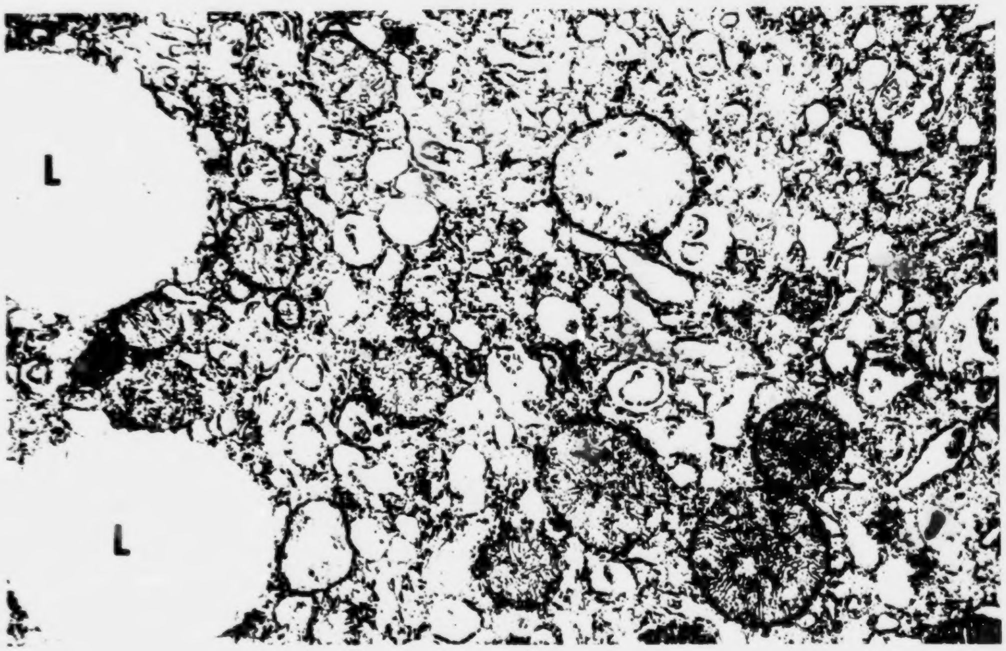

FIGURE 8. Hepatocytes from PCB-exposed fish show lipid droplets (L) and vesicular profiles of rough and smooth ER. X 24,000.

Subacute PCB exposure in the catfish resulted in several alterations in SER not seen in acutely-exposed fish liver. Tubular and vesicular profiles of SER (Fig. 9), nearly absent in control catfish hepatocytes, were increased in number and often accumulated in large aggregates. In addition, parallel stacks of SER in continuity with RER were observed (Fig. 10). Often, these patterns contributed to large whorls of SER in affected cells (Fig. 10). An additional whorl which is associated with a lipid droplet is shown (Fig. 11).

Thus, biochemical levels of induction following acute and subacute PCB exposure in the catfish correlated well with RER and SER content and morphology. Higher levels of MFOS induction were accompanied by greater increases in SER content and variations of SER morphology.

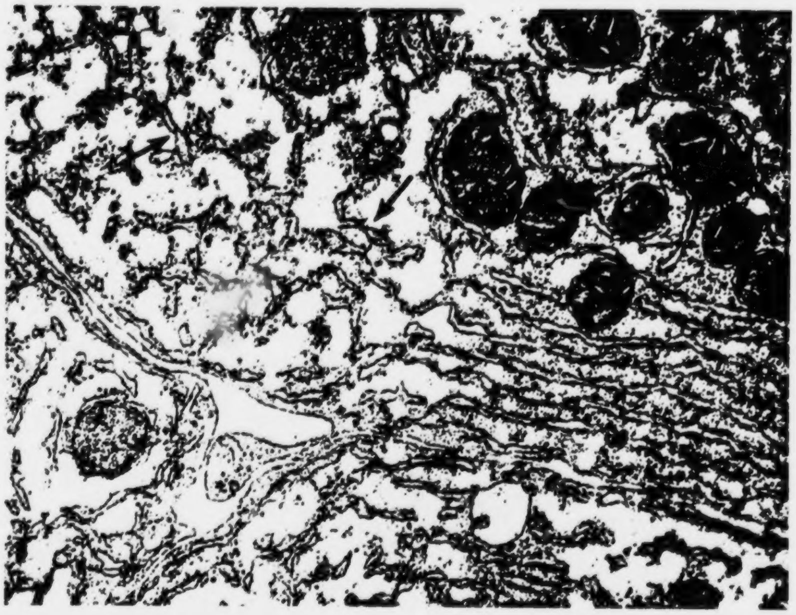

FIGURE 9. Tubular profiles of SER (arrows) are evident in hepatocytes of fish subacutely exposed to PCB. X 25,000.

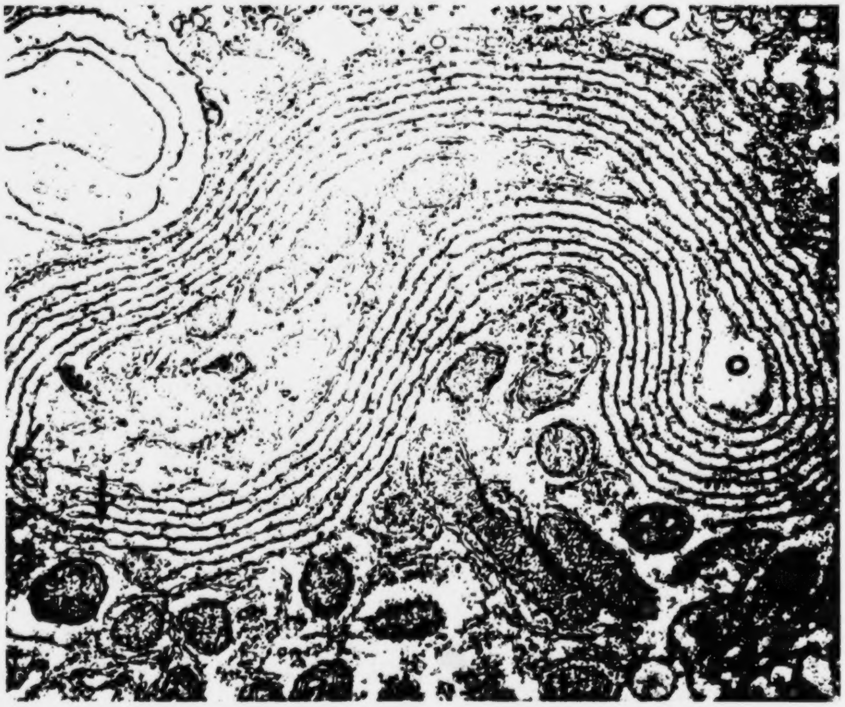

FIGURE 10. SER in continuity with RER (arrows) in a large membranous whorl. Subacute PCB-exposure. X 20,000.

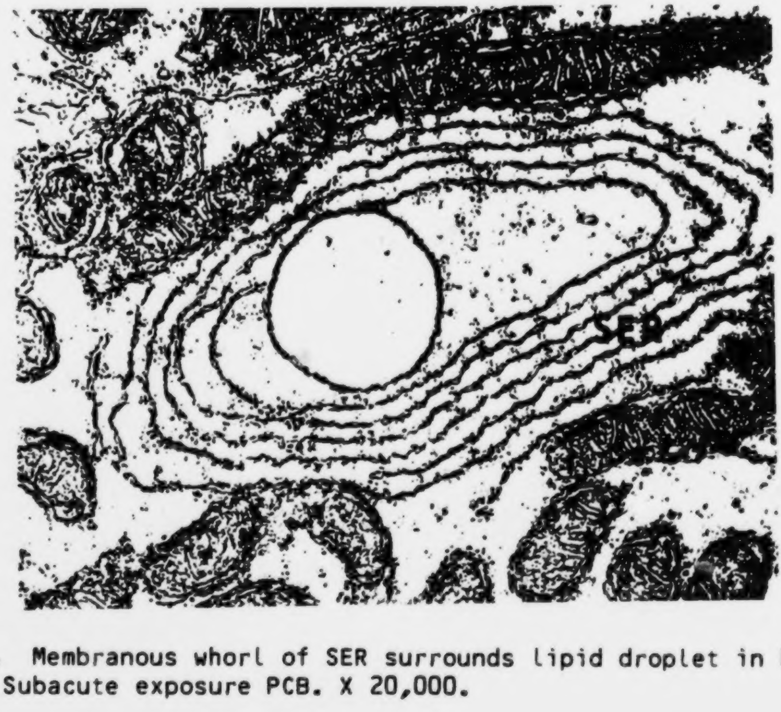

FIGURE 11. Membranous whorl of SER surrounds lipid droplet in hepatocyte of fish. Subacute exposure PCB. X 20,000.

3-MC

Acute 3-MC treatments caused significant induction of rainbow trout MFOS components (Table 3). Cytochrome P-450 increased to over 3 times control amounts. NADPH-cyt-c-reductase activity was elevated 2 X over controls. As in the PCB-exposed catfish, neither microsomal protein nor liver to body weight ratios were altered by 3-MC treatment.

Morphologically, acute MFOS induction correlated with apparent increases in RER (Fig. 12) as well as alterations in RER morphology. Parallel cisternal were often disrupted, dilated and vesiculated (Fig. 13). SER vesicles were also increased and were observed throughout cytoplasm (Fig. 13).

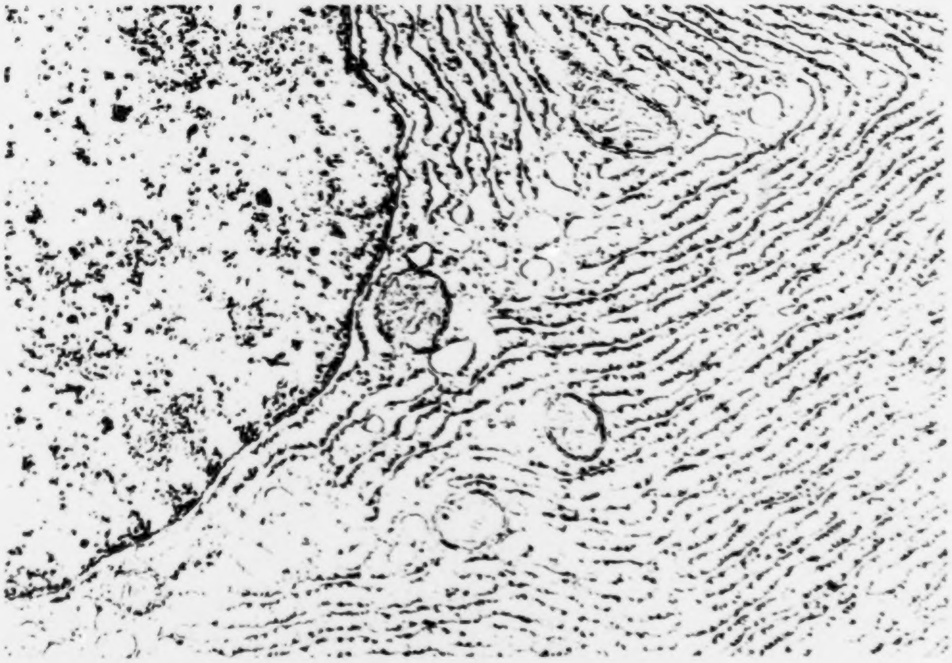

FIGURE 12. Perinuclear area of rough endoplasmic reticulum increase in hepatocyte of trout exposed to 3-methylcholanthrene. X 20,000.

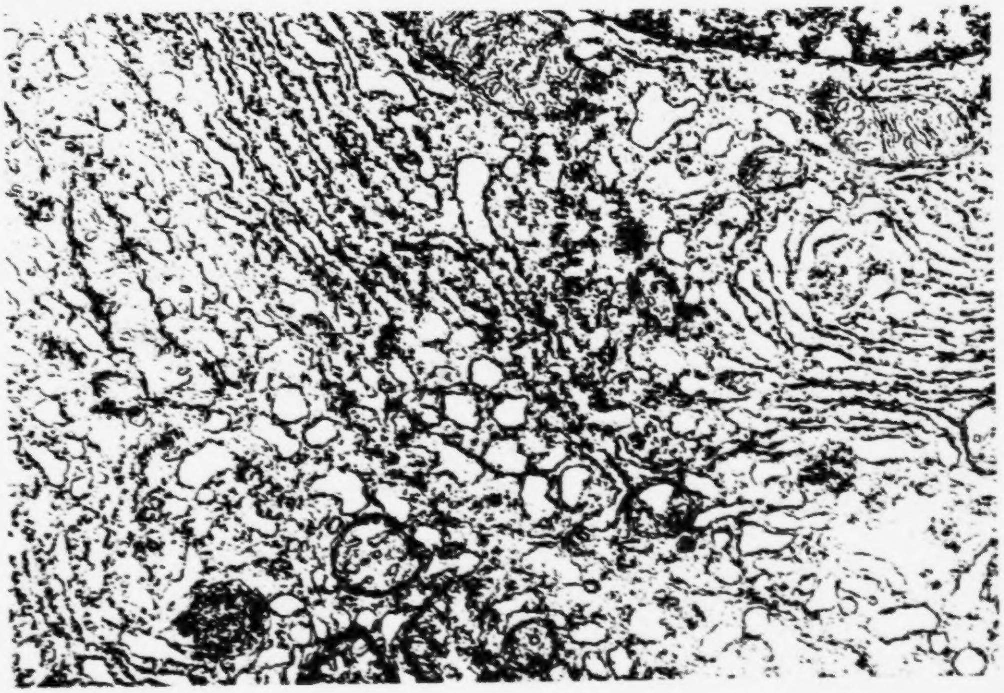

FIGURE 13. Portion of hepatocyte from 3-MC-treated trout. Numerous SER vesicles are in continuity with RER. X 20,000.

The ultrastructural alterations caused by acute PCB and 3-MC exposure involved RER content and morphology, although focal increases in agranular membranes were noted. The major response after 21 days of exposure to PCB was extensive proliferation of SER in the three distinct patterns previously described.

Scarpelli (111) reported similar findings in rainbow trout with the carcinogen, aflatoxin B . Acute response included increases in circular profiles of SER while more protracted exposure produced a greater morphologic response. In addition, membrane whorls were reported by the same investigator (112) in trout exposed to phenylbutazone. Based on our findings in the channel catfish exposed to PCBs and those of Scarpelli (111,112) in rainbow trout, it would appear that increases in alterations in RER and circular profiles of SER constitute the early morphologic responses of the teleost hepatocyte to inducers of the MFOS system. Extensive SER proliferation, commonly seen in the mammal after acute exposure, becomes apparent in the fish only after subacute or chronic exposure.

Caution should be taken in regard to the RER, since this organelle is abundant in hepatocytes of normal teleosts (111,114,115). However, since scant amounts of SER are seen in unexposed teleost liver (111,114), slight increases in this organelle as reported by Weis (116), in two species of fish exposed to DDT, are significant. It should be noted that the morphologic patterns of the endoplasmic reticulum in PCB and 3-MC exposed fish of this study correspond to functional patterns consistent with increased enzyme activity in numerous mammalian studies as reviewed by Smuckler and Arcasoy (117).

Characterization of Channel Catfish Liver AHH

The effect of pH on *in vitro* AHH activity is shown (Figure 14). Highest values were recorded between 7.0 and 7.2 for control, PCB, and 3-MC-pretreated microsomes. In control samples, activity decreased to only 59% of maximum when the pH was raised to 7.8.

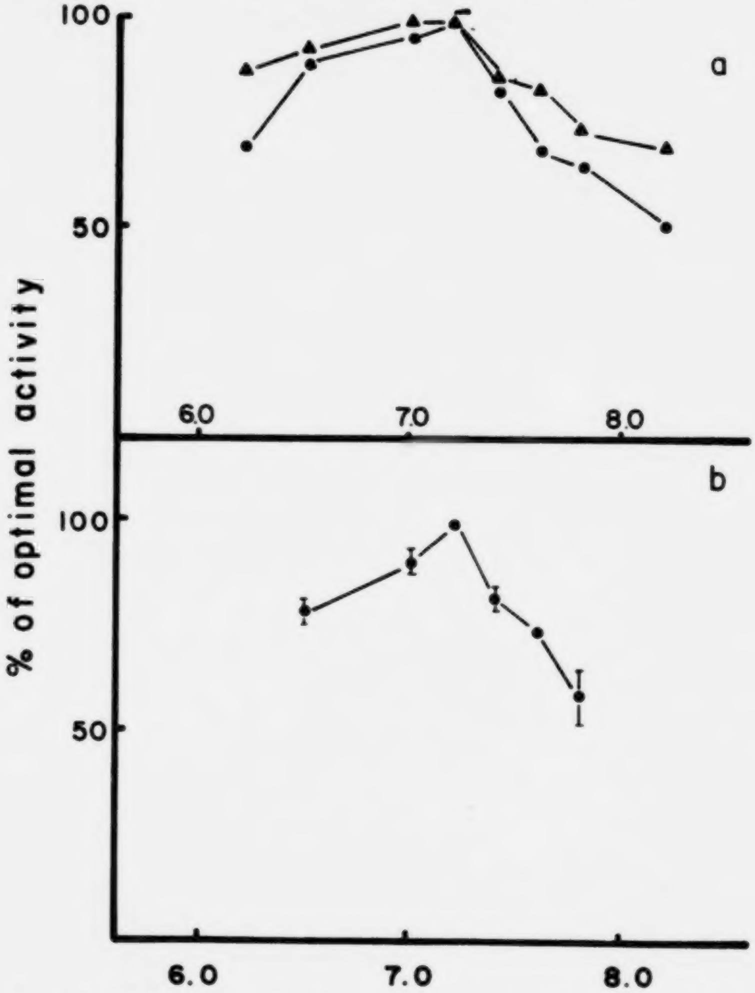

FIGURE 14. Effect of pH on *in vitro* AHH activity in liver microsomes from pretreated (a) and control (b) channel catfish.
a) Solid circle = PCB-pretreated fish; 4 daily i.p injections of 50 mg/kg.
Triangle = 3-MC-pretreated fish; 4 daily i.p. injections of 25 mg/kg.
AHH activity at pH 7.2 was termed 100%, and all other data points were normalized as a percent of that activity. Each data point represents the mean of 2 different experiments using triplicate samples for each point. Variation between replicate samples was less than 10%.
b) Control fish; 4 daily i.p injections of 0.5 ml corn oil.
Each data point represents the mean of 3 experiments using microsomal fractions "pooled" from 3 fish. Vertical bars represent X ± 1 S.D.

The optimum pH for the catfish liver AHH (7.0-7.2) was comparable although lower than the optimum pH of 7.5 reported for rainbow trout (68) and for sheepshead (71), but was identical to the pH range of 7.0-7.2 in scup (118). Studies ongoing in our laboratory using mammalian tissues find optima between 7.4 and 7.6. 3-MC or PCB-pretreatment did not change the pH range at which maximal activity was seen; rather, a flattening of the curve was noted.

The temperature dependence of AHH activity for control and pretreated microsomes is shown (Figure 15). Optimum for control enzyme was 30°C, and activity dropped to 70-86% at 35°C, 50% at 40°C, and 0% at 50°C. Fresh and frozen microsomal fractions were used in establishing temperature optima. In terms of specific activity, portions of the same microsomal fraction, assayed fresh and after freezing and storage for 24 hours at -80°C showed identical temperature optima. PCB and 3-MC-pretreated microsomes appeared more resistant to temperature increases. In the range of 30°-40°, no real peak existed.

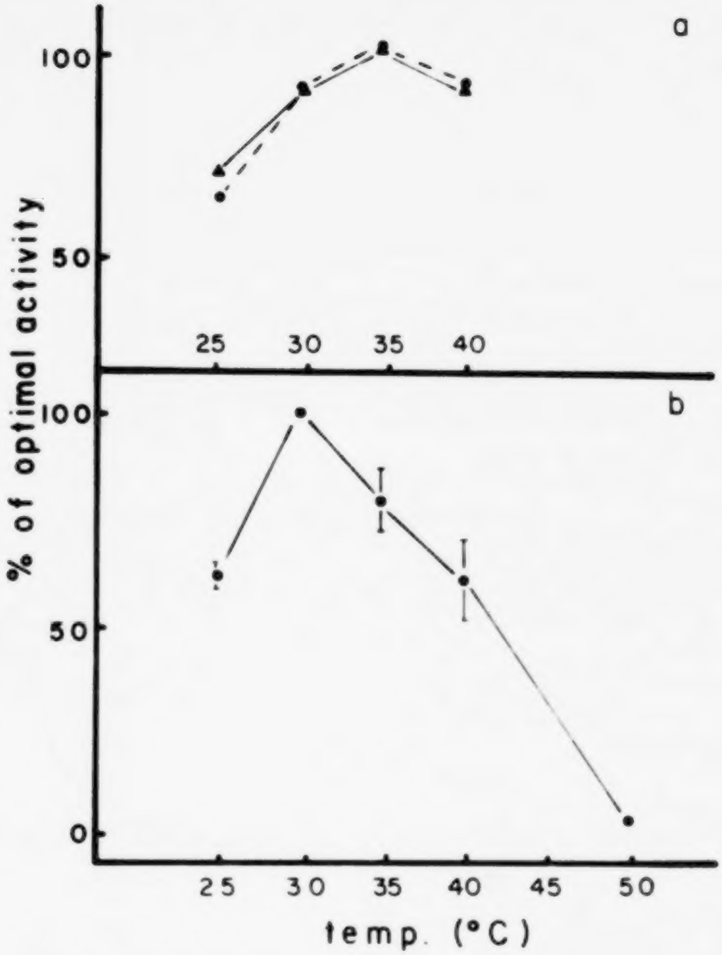

FIGURE 15. Effect of incubation temperature on in vitro AHH activity in pretreated and control liver microsomes from channel catfish.

a) Solid circle = PCB-pretreated fish (Exposure conditions identical to those in Figure 14).

Triangle = 3-MC-pretreated MC-pretreated fish (Exposure conditions identical to those in Figure 14).

AHH activity at 35° C was considered 100%. All other points were normalized as a percent of that activity. Each point represents average of 2 different experiments using triplicate samples for each point. Variation between replicate samples was less than 5%.

b) Control fish (as in Figure 14). AHH activity at 30° C was considered 100%. All other points were normalized as percent of that activity. Each point represents mean of 3 experiments using "pooled" microsomal fractions from 3 fish. Vertical bars represent ± 1 S.D. between the 3 experimental "pooled" samples. Activity at 50° C was negligible for all samples.

With respect to temperature, an optimum of 30° C was found for control catfish liver microsomes which corresponds to the temperature optima reported for rainbow trout (68), Finnish lake trout (66) and various marine species (64). A shift to a higher temperature range (30° C-40° C) followed treatment with 3-MC or PCB in the catfish. The induced system seems to have quite a broad temperature range with no sharp drop at higher temperatures such as that found in the trout (68). It is possible that the catfish, being a temperate water species, had a wider temperature optimum. James et al. (71) found a temperature optimum of 35° C in marine species native to coastal Florida, a much warmer environment. The shift, in the induced species, has not been reported before. Changes in membrane fluidity as well as enzyme could account for changes in temperature range.

The effects of microsomal protein concentration and time of incubation upon specific activity of AHH were determined. A linear relationship existed at microsomal protein concentrations up to and including 1.3 mg. The concentration routinely used was 0.1-0.2 mg protein per assay. AHH activity was linear through 20 min. of incubation. The above are similar to optimum conditions described by other investigators studying MFOS of various teleosts (64,66,67,69,71).

The effect of in vitro addition of benzoflavone (BF) upon AHH was studied in control, PCB and 3-MC-pretreated fish. Inhibition of activity was seen in 3-MC-pretreated samples at all ranges of BF tested (Figure 16). Inhibition ranged from 12% at .001 mM BF to 76% at 1 mM BF. PCB treatment left AHH less sensitive to BF inhibition at most concentrations than did 3-MC treatment. In control samples, .001 mM BF had no effect on AHH levels; however, at concentrations of .01mM, up to 200% enhancement of activity was seen. Enhancement decreased with increasing BF concentrations, until at 1 mM BF, 22% inhibition was seen. No enhancement was seen in samples from either treated group at all concentrations tested.

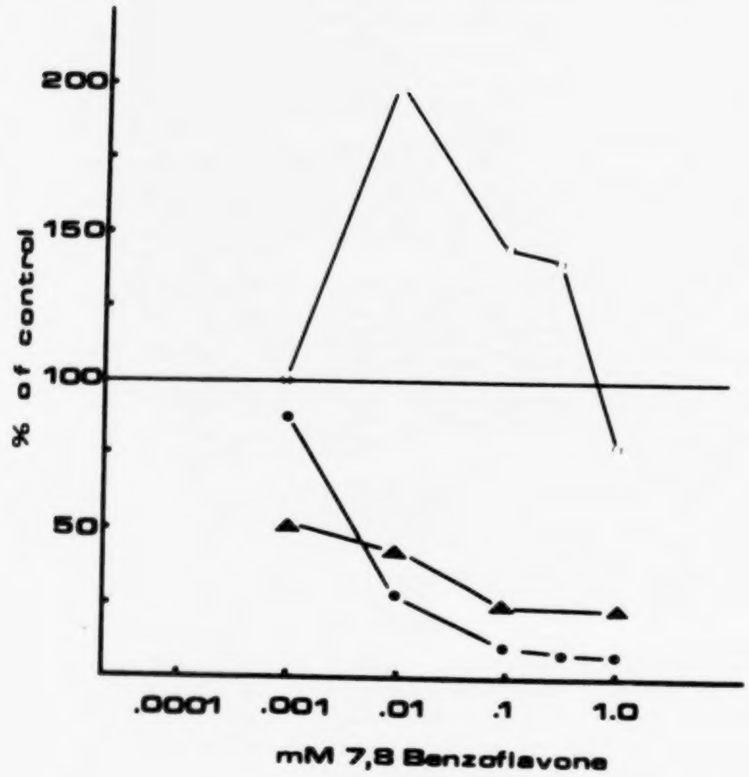

FIGURE 16. Effects of 7-8 benzoflavone concentration on *in vitro* AHH activity. Microsomes from control liver = open circles; microsomes from 3-MC-treated livers = solid circles; microsomes from PCB-pretreated liver = triangles.

Control equals the same sample without BF. Data illustrated are from a single representative experiment. Triplicate assays were performed with variation between replicates less than 10%.

BF inhibits what is usually termed cytochrome P-448, that form of microsomal cytochrome P-450 which arises following PAH treatment in the rat. The effect of BF on in vitro AHH was studied in untreated and dimethylbenzanthracene (DMBA) pretreated little skates by Bend et al. (67). Higher concentrations of BF (0.1-1.0 mM) caused nearly 3-fold stimulation of AHH in control animals, but inhibited activity in induced animals by 80%. James et al. (71) also reported a similar effect of BF on hepatic AHH activity in the sheepshead. In that study, all concentrations of BF tested (10^{-7} M-10^{-3} M) enhanced control AHH activity but inhibited AHH in 3-MC-pretreated fish. Higher concentration of BF (0.1-1.0 mM) caused about 4-fold stimulation of AHH in control animals, and inhibited activity in induced animals by about 60%. In rat liver, Weibel et al. (119) found that the in vitro addition of BF inhibited AHH in rats treated with 3-MC while activity was enhanced in control and phenobarbital-treated animals. Our data using catfish liver microsomal fraction correspond to findings in little skates (Bend et al. (67), sheepshead (71), and rat liver (119). Inhibition is seen following PCB and 3-MC-treatment while enhancement occurs only in control animals. Wiebel et al. (119) did not report inhibition in control rat liver at BF concentration of 1 mM; however, that experiment did not include such high concentrations. The enhancement in control little skates was lower at 1 mM BF (67); however, no decrease was seen in AHH activity in sheepshead at this concentration (71).

Studies thus far have demonstrated that two forms of AHH can be distinguished, one in control fish liver microsomes and the other in microsomes from MC or PCB-pretreated fish. The enzymes are distinguished by the differential effect of BF on AHH. This individualism may be due to a different affinity of BF for the enzymes. The binding sites themselves could vary, or membrane structures allowing access to the sites could differ. An allosteric activation of the enzyme complex could be responsible for the BF-produced enhancement of AHH in untreated animals (119). BF could bind to cytochrome P-450 and change its reduction and reoxidation rate. BF has also been found to protect cells grown in culture from DMBA toxicity (120), as well as to inhibit tumorigenesis induced by DMBA in mouse skin (121). BF inhibition, at the concentration studied, can be used in catfish only as a qualitative index of induction. Other species differ in their response to BF.

The findings of Ahokas et al. (83) and Stegeman and Binder (118) in different species are contrary to those found in the catfish. Ahokas et al. (83) observed 88% inhibition of AHH by 0.1 mM BF in untreated Finnish lake trout. Likewise, Stegeman and Binder (118) reported 80-90% inhibition of AHH activity by 0.1 mM BF in control Scup. Both investigators reported very high AHH levels in these species. The BF sensitivity and high AHH values in control animals suggest interspecies variation in native P-450 systems, as well as the possibility that the control animals found to be BF sensitive may have been induced in the wild by aquatic pollutants. Fish used by Stegeman and Binder (118) were wild; however, those used by Ahokas et al. (83) were hatchery reared. Of interest here is the fact that James et al. (71) found 8 out of 96 untreated sheepshead and 1 out of 6 untreated Southern flounder (from the wild) to have higher than usual AHH activities, which were inhibited in vitro by BF. The cause of this apparently induced condition was not known.

From the data discussed above, the AHH in catfish appears to be in some ways similar to that in mammalian and other fish systems. Both 3-MC and PCB treatment in vivo cause the behavior of the AHH enzyme to change. Characteristically, the induced enzyme is similar to the control enzyme with respect to pH optimum, but differs in temperature optimum, and BF sensitivity (at the same concentration). Whether this is due to new protein formation or not is debatable; however, changes in the membrane composition should be considered. Perhaps treatment exposes more enzyme or a different form of enzyme, hidden before, to the substrate (34).

BP Induction of Channel Catfish AHH

BP, adminstered as a single i.p. injection of 100 mg/kg b.w. in corn oil, induced its own in vitro metabolism within 48 hrs. in livers of 3 of 4 channel catfish (Figure 17-A). Differences in response of exposed individuals was apparent with AHH values ranging from 120 to 350% of control values. Taken as a group, the mean value for exposed fish was 1.90 ± 1.41 compared to 0.50 ± 0.04 for controls ($p < .025$).

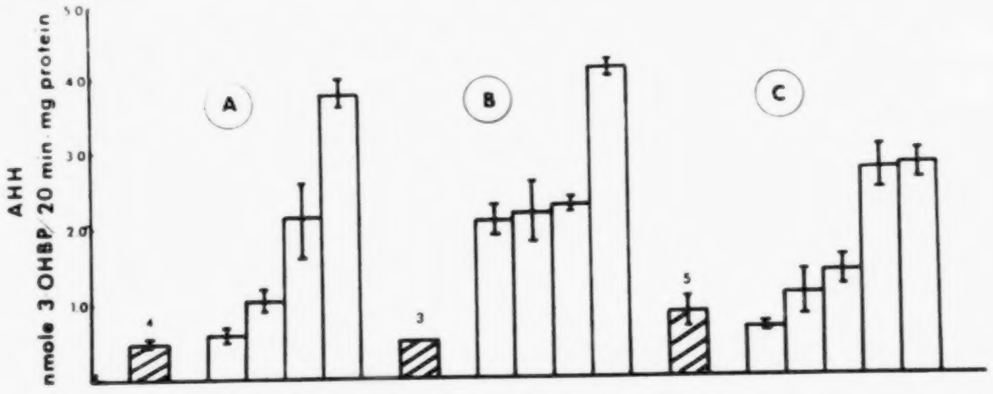

FIGURE 17. Effects of BP on hepatic AHH activity in channel catfish. Slashed bars represent the average of control fish ± 1 S.D. between animals. Numbers over the bars represent the number of fish in the experiment. Clear bars represent individual treated fish ± S.D. for triplicate assays on each animal. Treatment: (A) one i.p. injection of 100 mg BP/kg b.w., sacrificed after 48 hrs. (B) six daily injections of 25 mg BP/kg b.w., sacrificed after 24 hrs. (C) one gastric intubation of 100 mg BP/kg b.w., sacrificed after 14 days.

When BP was administered as 6 daily i.p. doses of 25 mg/kg (Figure 17-B), induction of AHH was seen and individual variation among fish was reduced. The mean AHH value in exposed fish was 2.67 ± 0.97. This is, on the average, a 513% increase over the control mean of 0.52 ± 0.01. These values were significantly different ($p < 0.01$). When BP was given as a single gastric intubation, induction of AHH occurred in 80% of treated fish (Figure 17-C). Inter-individual variation was apparent. The mean for exposed fish (1.73 ± 0.95) was 208% of control values and was significantly different ($p < .025$).

3-MC Induction of Channel Catfish AHH

The effects of 5 daily i.p. injections of 20 mg 3-MC/kg body weight upon channel catfish liver AHH are shown (Figure 18). All fish exposed in this manner exhibited induction with the individual response ranging from 10 to 25 times control values. The experimental mean of 4.20 ± 1.73 was statistically different from the control mean of 0.27 ± 0.12 ($p < .01$).

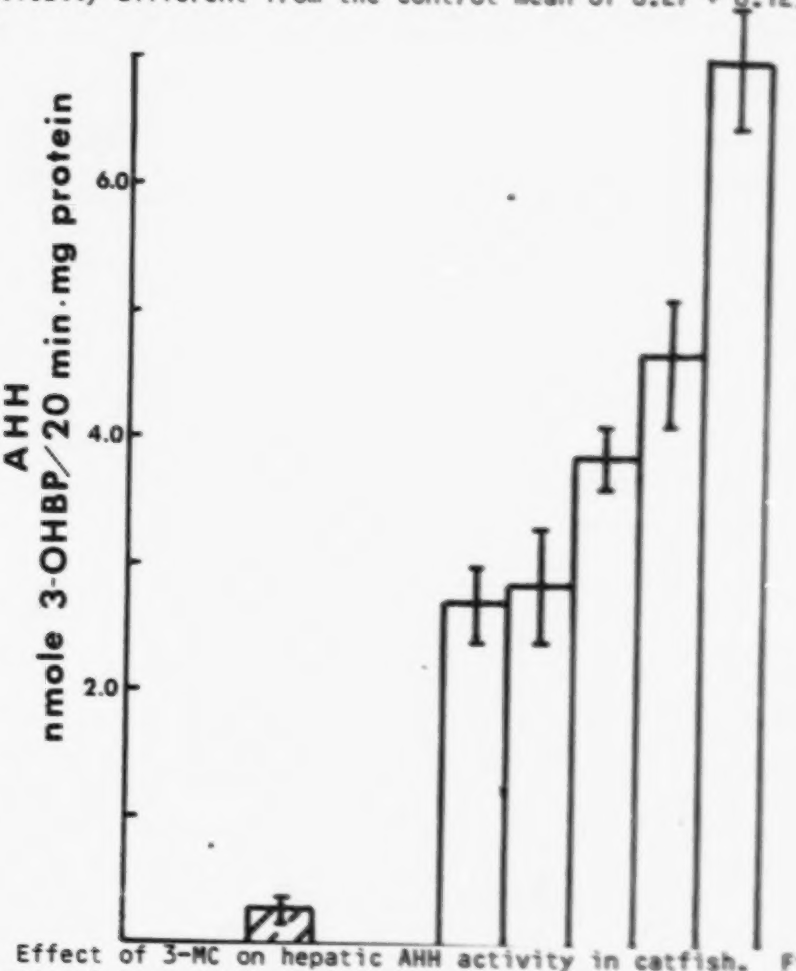

FIGURE 18. Effect of 3-MC on hepatic AHH activity in catfish. Five fish were injected on 5 consecutive days with 20 mg 3-MC/kg b.w., and sacrificed after 24 hrs. The slashed bar represents the mean of 3 control fish ± 1 S.D. between animals. Clear bars represent individual treated fish ± 1 S.D. for triplicate assays.

When 3-MC was administered as 3 gastric intubations totaling 57 mg/kg body weight (Fig. 19), livers from all exposed fish showed induction ranging from 300 to 850% of control values ($p < .01$).

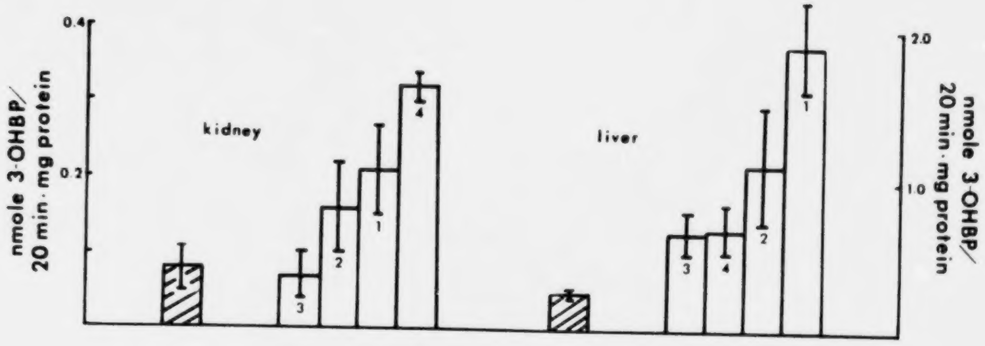

FIGURE 19. Effect of 3-MC on kidney and liver AHH in catfish. Four fish were given 3 gastric intubations of 25 mg 3-MC/kg b.w. and sacrificed after 96 hrs. Slashed bars represent means of 4 control fish ± 1 S.D. between animals. Clear bars represent individual treated fish ± 1 S.D. for triplicate assays on each fish. Numbers inside the clear bars identify the individual fish.

AHH values for kidneys from the same fish showed induction by 3-MC in 3 of 4 fish. When taken as a group, the average AHH in kidney for exposed fish was 0.19 ± 0.11 compared to 0.08 ± 0.03 in controls ($p < .025$). AHH activity in kidney was approximately 20% of the activity in liver. A high value of AHH in liver did not correlate directly with values for the same fish in the kidney. The fish with the lowest liver AHH also showed the lowest kidney AHH.

AHH Induction in Other Teleosts

Preliminary findings have been made on the effect of exposure to PAH on liver AHH in additional species. Brown bullheads were fed trout chow containing 100 mg 3-MC/kg diet at 1% of the biomass for 21 days. When compared to controls, AHH of exposed fish was 2.5 times higher. Bluegill sunfish, administered 3 daily i.p. injections of 3-MC in corn oil (25 mg/kg), showed a 3-fold increase over control AHH at day 4. Mummichogs fed 200 ppm BP or 3-MC in the diet at 1% of biomass for 30 days had AHH levels of 1.5 and 1.8 X control respectively.

Table 5 summarizes partial data on selected MFOS components in brown bullhead, fathead minnows and bluegills after PAH. These data represent initial findings and are included only to show some components of the MFOS. Continued investigation of these species is being performed.

TABLE 5. EFFECTS OF PAH ON HEPATIC MICROSOMAL MFOS OF TELEOSTS

	BROWN BULLHEAD		FATHEAD MINNOW		BLUEGILL	
	Control	BP[a]	Control	BP[b]	Control	3-MC[c]
1. NADPH-cytochrome-c-reductase	15.78(4)	17.8(4)	23.06(11)	130.0(7)	65.12(2)	32.84(2)
2. Cytochrome P-450	.120±(4)	0.061±(4)	0.175(11)	ND	.160(2)	.172(2)
3. Aminopyrine demethylase	2.15(4)	3.27(4)	1.481(7)	3.901(7)	3.30(2)	3.60(3)
4. Microsomal protein	20.21(4)	14.04(4)	11.32(11)	8.67(7)	12.4(2)	13.47(2)
5. Liver/body weight X10-2	2.0±0.06 (4)	2.5±0.06 (4)	1.6±0.06	2.7±0.03	0.96(2)	0.94(2)

(#) - # of livers in pooled sample.
1) nmoles cyt-c-reduced/min/mg protein.
2) nmoles/mg proteins.
3) nmoles HCHO formed/min/mg protein.
4) mg/g liver.
a) Fed 3 weeks 100 ppm BP.
b) Fed 100 ppm BP.
c) i.p. 25 mg 3-MC/kg bw as 3 daily injections.
ND - not determined.

Dose-Response Study of 3-MC in Channel Catfish MFOS

AHH, NADPH-cytochrome-c-reductase, and cytochrome P-450 were measured to determine the effect of increasing doses of 3-MC on the MFOS in channel catfish. Table 6 summarizes the results.

BP metabolism, as measured by AHH, was enhanced by prior exposure to 3-MC at all concentrations. No direct relationship was seen between dose and induction at these concentrations. Four injections of 10 mg/kg b.w. caused an overall 17-fold increase in AHH. No appreciable difference in NADPH-cytochrome-c-reductase could be seen at this dose. Increases in cytochrome P-450 were seen in some but not all fish tested. At the intermediate dose range (25-50 mg/kg b.w.), a 20-fold increase in AHH was seen. Again, no difference in NADPH-cytochrome-c-reductase was seen; however, mean values for cytochrome P-450 were higher than the control values. The 100 mg/kg dose brought about a 22-fold increase in AHH, a 1.5-fold increase in mean values for NADPH-cytochrome-c-reductase, and a 2-fold increase in cytochrome P-450. Statham et al. (82) found no further increase in AHH at concentrations higher than 20 mg/kg b.w. To establish a true dose response curve, lower concentrations of 3-MC must be used.

For AHH and NADPH-cytochrome-c-reductase, the inter-individual variation, as observed by the standard deviations, increased with the dose. This interindividual variation in AHH has been observed in sheepshead (69) and rainbow trout (72). Pedersen et al. (72) administered several doses of 3-MC to one strain of trout and found highly variable responses between individuals at each dose and a slight positive correlation between dose and increasing hepatic AHH activity. The deviation between control catfish was quite small in all cases. Microsomal protein was not changed by the four day exposure to any level of 3-MC.

Duration of Effect of 3-MC Treatment on Catfish AHH

When constant dose (25 mg 3-MC/kg b.w.) was maintained for 4 days and fish were then killed daily up to one week after exposure, it was possible to determine the duration of effect. These data are presented in Table 7.

AHH was increased dramatically by 1 day after the cessation of exposure. At day 4 following cessation of exposure, the AHH activity was 24 times the control mean, and by day 7 after treatment, AHH was 19-fold the control mean. The deviation between individual fish decreased markedly as the time of exposure lengthened. The highest values for NADPH-cytochrome-c-reductase and cytochrome P-450 were seen at day 4. At this time, the mean cytochrome P-450 estimation was 2.2 times over the control value. By day 7, cytochrome P-450 values were only slightly higher than controls. Statham et al. (82) found AHH to reach a maximum at 48 hrs. after injection of 3-MC in trout.

Duration of effect studies with 3-MC have been reported on sheepshead and the Atlantic stingray by Bend et al. (67,69). Animals were treated

TABLE 6. DOSE RESPONSE STUDY OF 3-MC ON CHANNEL CATFISH HEPATIC MFOS

No. of Fish	*Treatment	AHH nmoles/20 min/mg Protein	NADPH-cytochrome-c-reductase S.A. nmoles/min/mg Protein	Cytochrome P-450 nmoles/mg Protein	Microsomal Protein mg/ml
4	Control Corn Oil	0.21±.03	14.07±1.7	.142±.03	3.32±.5
3	10 mg	3.68±1.03	15.2±3.1	.233±.1	3.54±.8
3	25 mg	4.37±1.17	15.9±2.7	.251±.09	2.57±.5
3	50 mg	4.34±2.10	15.6±7.6	.175±.06	4.42±.3
3	100 mg	4.62±2.18	20.9±11.7	.241±.07	3.49±.2

* 4 consecutive daily i.p. injections of 3-MC/kg b.w.; values are mean ± 1 S.D.

TABLE 7 DURATION OF EFFECT OF IN VIVO 3-MC TREATMENT ON HEPATIC MFOS OF CATFISH

No. of Fish	*Treatment	**Day	AHH nmoles/20 min/mg Protein	NADPH-cytochrome-c-reductase nmoles/min/mg Protein	Cytochrome P-450 nmoles/mg Protein	Microsomal Protein mg/ml
4	Control Corn Oil		.21+.03	14.07±1.7	.142±.03	3.32±.5
3	25 mg 3-MC/ kg b.w.	1	4.37±1.17	15.9±2.7	.251±.09	2.57±.5
3		2	4.43±.83	15.7±2.9	.201±.06	3.95±.6
3	"	4	5.05±.59	17.2±2.9	.314±.10	4.97±.7
3	"	7	4.05±.44	15.7±1.7	.177±.03	5.11±1.1

* 4 consecutive daily i.p. injections.
** Day of sacrifice after treatment ended.
Values = mean ± 1 S.D.

with two i.p. injections of 20 mg 3-MC/kg b.w. Cytochrome P-450 content in control sheepshead was 0.28 nmoles/mg protein. Six days after exposure it peaked at 0.54 nmoles/mg protein (1.8 x control). AHH increased to 10-fold over control values by 3 days after treatment and dropped off somewhat by day 6. As in catfish, the AHH increased prior to cytochrome P-450 content.

Bend et al. (69) saw a marked species difference in response to equivalent doses of 3-MC between the Atlantic stingray (a cartilaginous fish) and teleosts. The former showed no change in either cytochrome P-450 or AHH after 3-MC treatment. Extensive differences were also seen in AHH activity as well as aniline hydroxylase and nitro reductase in 6 strains of rainbow trout (72). Philpot and Bend (122) found this species difference to apply to rabbits, which also showed no increase in AHH after 3-MC treatment.

The flux in inter-individual variation among treated animals (most evident in AHH determination) is of interest. Factors which cause inter-individual variation are sex, degree of hormonal stimulation (74), and differential rates of uptake from sites of deposition in individual fish. The longer 3-MC remains in the animal the closer AHH values become among the individual animals of the group. 3-MC is quite lipophilic and it is possible that the compound remains in body fat of some fish longer than in others.

Seasonal variation of MFOS enzyme activity has also been noted in fish by Dewaide and Henderson (123). After collecting fish throughout the year, activities of aminopyrine demethylase and aniline hydroxylase were higher in summer months and lower in winter. After holding fish under laboratory conditions, these differences vanished. Seasonal cycles in fish with changes in metabolic and endocrine activity occur and could be at least partially due to factors such as temperature and daylight. Fish are poikilotherms and, therefore, rates of metabolism and chemical uptake are temperature dependent (124). Differences could also arise from food intake and exposure to foreign compounds which induce the MFOS (123). Since fish are more activie in the summer, (123) a higher concentration of enzyme inducing chemical agents due to a lower water level may be one reason for seasonal changes.

Annual reproductive cycles are also accompanied by extremes in metabolism and male/female differences (74). Stegeman and Chevion (74) reported differences in the hepatic cytochrome P-450 of gonadally mature male and female trout. This microsomal cytochrome was not catalytically equivalent in males and females, as was illustrated by differences in enzyme activity, the Soret peak for reduced CO-bound cytochrome P-450, and BF sensitivity. Fluctuating levels of testosterone, a common steroid in fish (74), could result in these differences. Testosterone stimulates MFOS activity in male rats (125).

Age and sex should not have played a part in inter-individual variation in the catfish studies, since all fish were of the same size, and sexually immature. They were kept in a controlled environment and not subjected to seasonal and temperature changes. Inter-individual variation, therefore, may be assumed to be caused primarily by genetic differences between individual fish.

As was discussed earlier, the hepatic P-450 system induced by 3-MC in the rat is termed P-448, due to a 2 nm shift in the absorption maximum from 450 nm to 448 nm (126). Throughout this study on the catfish, the cytochrome P-450 was monitored carefully, and no shift after 3-MC-treatment was seen. The absorbance maximum for the CO-bound reduced cytochrome P-450 in both control and MC-pretreated channel catfish was 450 nm. This is similar to reports in fish by Bend et al. (69), Pedersen et al. (72), and Statham et al. (82).

Bend et al. (67) saw an 8-fold increase in AHH in the little skate after two oral treatments of 3-MC (50 mg/kg b.w.), and reported an absence of a 2 nm shift in the cytochrome P-450 peak. Statham et al. (82) and Pedersen et al. (71) reported similar increases in AHH in trout, a 50% increase in cytochrome P-450 content (82), and no shift in the reduced CO-bound cytochrome P-450 after 3-MC treatment. This suggests a catalytically different form of cytochrome P-450 being synthesized in catfish, little skate, and trout in response to PAH with the same absorbance maximum when reduced and CO-bound as the native cytochrome P-450.

The microsomal MFOS of rat liver was studied by Alvares et al. (126) after treatment with 25 mg 3-MC/kg b.w., i.p., for 4 days. Animals were sacrificed 24 hours after the last dose. This design compares well to our catfish study. Microsomal protein in 3-MC-pretreated rats was 106% of control, not a substantial change. NADPH-cytochrome-c-reductase was 108% of control, and cytochrome P-450 was increased to 237% of control values.

Microsomal protein concentration in catfish (Table 7) appeared to increase in the fish killed 4 and 7 days after treatment ended; however, no clear cut correlation between induction and protein concentration was seen. The mechanism for induction in fish has not yet been explained. New enzyme synthesis or enzyme modification is one possibility; however, changes in the membrane composition or conformation should also be considered.

From the above, it is evident that NADPH-cytochrome-c-reductase does not increase immediately after PAH treatment as does AHH and cytochrome P-450, but can increase after time.

These studies have reported on *in vitro* BP metabolism following treatment with a PAH. The need remains for the *in vivo* measurement of effects of such compounds as has been performed with one PAH and methylnaphthalene (82).

HPLC Results

Several different gradient systems were tried before deciding on the one that was most satisfactory as described in Materials and Methods. Figure 20 is a tracing of pure metabolite standards obtained with the described gradient system and Whatman column by monitoring the aborbance at 254 nm.

Good separation and resolution of metabolites was obtained. Of the three diols, 9,10-diol BP material was left out here; line should read as follows: three diols, 9,10 diol-BP, being most polar, comes off the column first, followed by 4,5 diol-BP and 7,8 diol-BP. The hydroxy derivatives, 9-OH BP and 3-OH BP, follow the diols, and unmetabolized BP is eluted last. Quinones appear between the hydroxy region and the BP peak. Pezzuto et al. (127) obtained very similar separation of BP metabolites using an identical Whatman column. Retention times are given in Table 8.

A Permaphase ODS column was tried, but the longer column (1 meter) gave poor resolution, peak tailing, and inconveniently long retention times. This is probably due to the lack of temperature control on the Varian instrument. If a higher than ambient temperature (50°C) could have been maintanined, the retention times would have been much shorter and resolution and tailing would probably not have posed a problem. Under our conditions the 25 cm Whatman column proved to be most satisfactory.

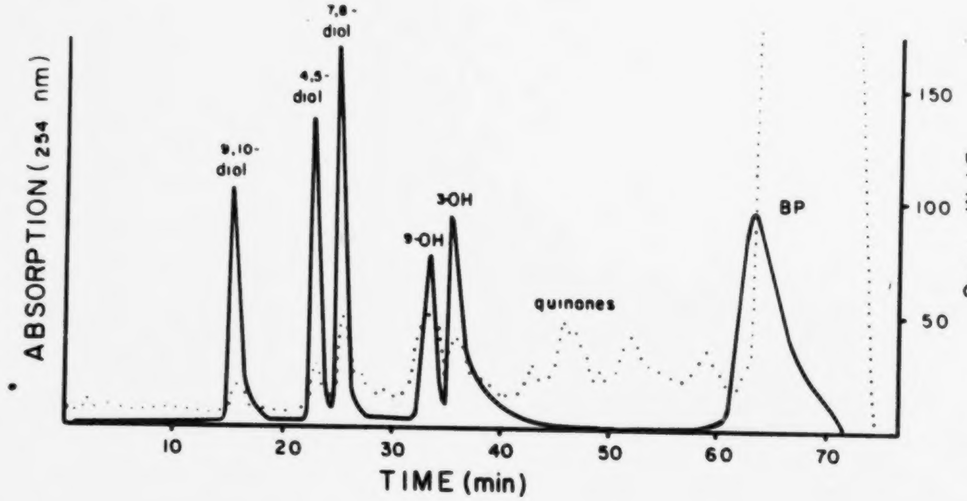

FIGURE 20. HPLC chromatograph of BP metabolites. The solid line is a tracing of pure metabolite standards (obtained from IITRI) as detected by UV absorption at 254 nm. The dotted line represents rat liver microsomal metabolites of ^{14}C-BP internal standard. Retention time in minutes is on the X-axis.

TABLE 8. RETENTION TIMES (R.T.) OF BP METABOLITES SEPARATED BY HPLC

Metabolite	R.T.
9,10-diol	15 min
4,5-diol	23 min
7,8 diol	25 min
9-OH	35 min
3-OH	36 min
BP	63 min

Column: Whatman Partisil (PXS 10/250 DS), 25 cm in length, inside diameter = 4.6 mm.
Gradient: 25% 30:70 methanol:water to 100% 70:30 methanol:water at 3%/min.
Flow Rate: 1.2 ml/min.
Metabolite Standards were obtained from IIT Research Institute, Chicago, IL.

Table 9 provides a summary of results in terms of percent metabolism of BP into the various metabolites. Three different control and 3-MC-pretreated catfish were used to obtain this data. The 3-MC-pretreated catfish showed an apparent increase in activity toward the formation of all metabolites. Control and 3-MC values for 9,10-diol BP and 7,8-diol BP were significantly different by the T test ($p < 0.05$). Of the total radioactivity added per assay, 3-Mc-pretreated microsomes metabolized 12-28% (3.75-4.8 uCi) and control microsomes metabolized 14-22% (3.0-5.6 uCi). The PCB-pretreated catfish also show an increase in 9,10-diol BP and 7,8-diol BP although total metabolism of BP was only 3% (0.43 uCi). 3-MC-pretreated trout also showed increases in 9,10-diol BP and 7,8-diol BP over control. Control trout metabolized only 3% of the labeled BP (0.64 uCi), while 3-MC-pretreated trout metabolized 13% (2.78 uCi).

TABLE 9. SUMMARY OF BP METABOLITE FORMATION AS % OF METABOLISM

METABOLITE	CATFISH					TROUT	
	Control	+BF	3-MC	+BF	PCB	Control	3-MC
BP - 9,10-diol	1.97 ± .15	3.9	3.77±1.07	4.0	9.5	1.7	5.9
BP - 4,5-diol	3.77 ± .93	14.0	6.03±4.03	1.9	2.6	1.8	1.0
BP - 7,8-diol	7.47 ± 1.27		1.25±1.09	5.4	12.6	3.3	14.0
9-OH - BP	14.5 ± 2.3	20.0	19.2±2.85	5.7	19.6	13.0	10.6
3-OH - BP	13.17 ± 3.17	33.0	13.6±4.07	12.0	5.5	8.5	12.6
quinones	12.5 ± 1.32	22.0	16.4±3.38		13.8	45.0	22.0
total BP metabolized	14-22%	28.8%	12-28%	10%	3%	3%	13%
AHH nmoles/20 min/mg protein	0.6	1.0	6.08	0.7	2.71	0.54	7.37

Data from three individual fish are given for control and 3-MC-pretreated Catfish (50 mg/kg b.w. x 4 days). The concentration of BF when added was 0.1 nM. Data for trout represent "pooled" microsomal fractions from 4 control and 3-MCtreated fish (50 mg/kg b.w.).

The *in vitro* addition of 0.1 nM BF to microsomes from untreated catfish caused an increase in all metabolites over the straight control preparations, plus a 28.8% metabolism of BP 97.9 uCi, corresponding to the enhancement of AHH discussed earlier. The presence of *in vitro* BF in 3-MC-pretreated microsomal preparations markedly inhibited formation of most metabolites and AHH activity. Total metabolism was only 10% (2.76 uCi).

Figures 21 and 22 are HPLC chromatographs for control vs. 3-MC-pretreated catfish and control vs. 3-MC-pretreated trout respectively. Figure 23 represents a PCB-pretreated catfish. These tracings are superimposed to visualize the increase in peak height, seen especially in 9,10 diol BP and 7,8-diol BP peaks in both species of fish.

A shoulder just prior to the major 7,8-diol-BP peak usually appears in both control and treated fish samples. Multiple hydroxy peaks can also be seen in 3-MC-pretreated catfish and trout. These points differ with regard to the rat liver HPLC tracing (fig. 20). It should be noted that the 9-OH-BP and 3-OH BP peaks also contain other hydroxy derivatives such as 1-OH BP and 7-OH BP.

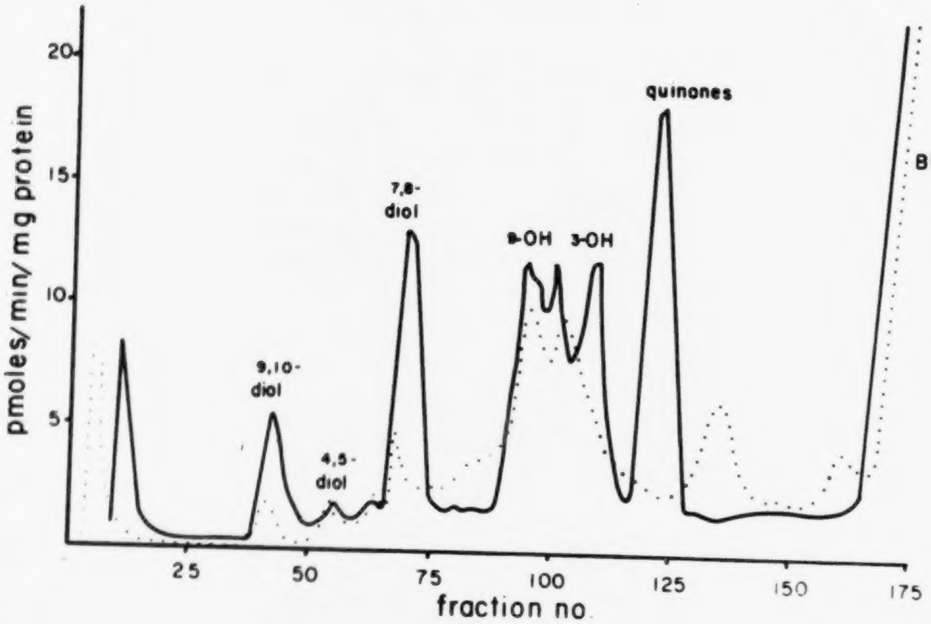

FIGURE 21. Radioactivity in HPLC eluate following incubation of ^{3}H-BP with liver microsomal fraction from control and 3-MC-treated catfish. Dotted line represents control catfish liver microsomes, and solid line represents 3-MC-pretreated catfish liver microsomes.

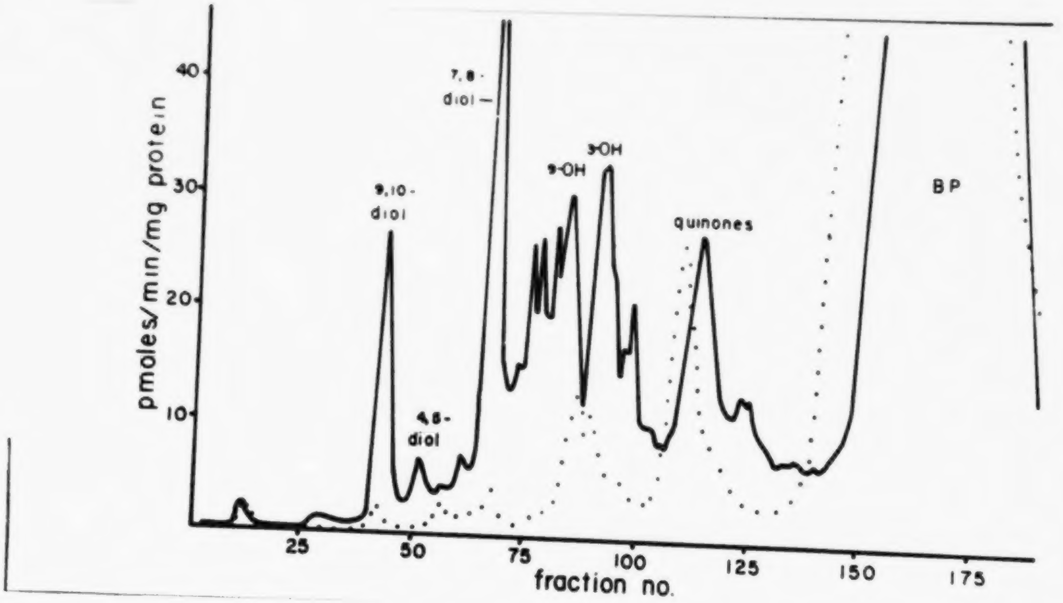

FIGURE 22. Radioactivity in HPLC eluate following incubation of ^{3}H-BP with liver microsomal fraction from control and 3-MC-pretreated trout. Dotted line represents control trout liver microsomes, and solid line represents the 3-MC-pretreated trout liver microsomes.

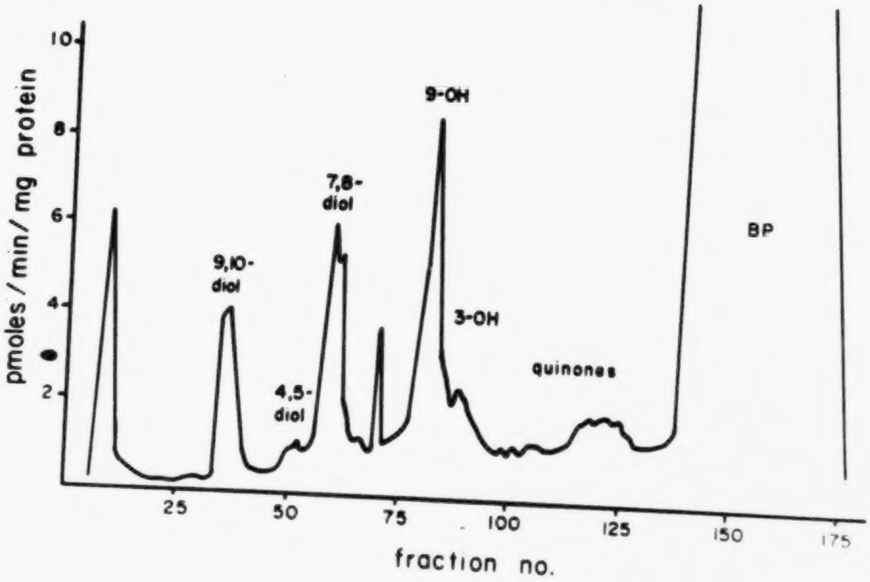

FIGURE 23. Radioactivity in HPLC eluate following incubation of ^{3}H-BP with liver microsomal fraction from PCB-treated catfish.

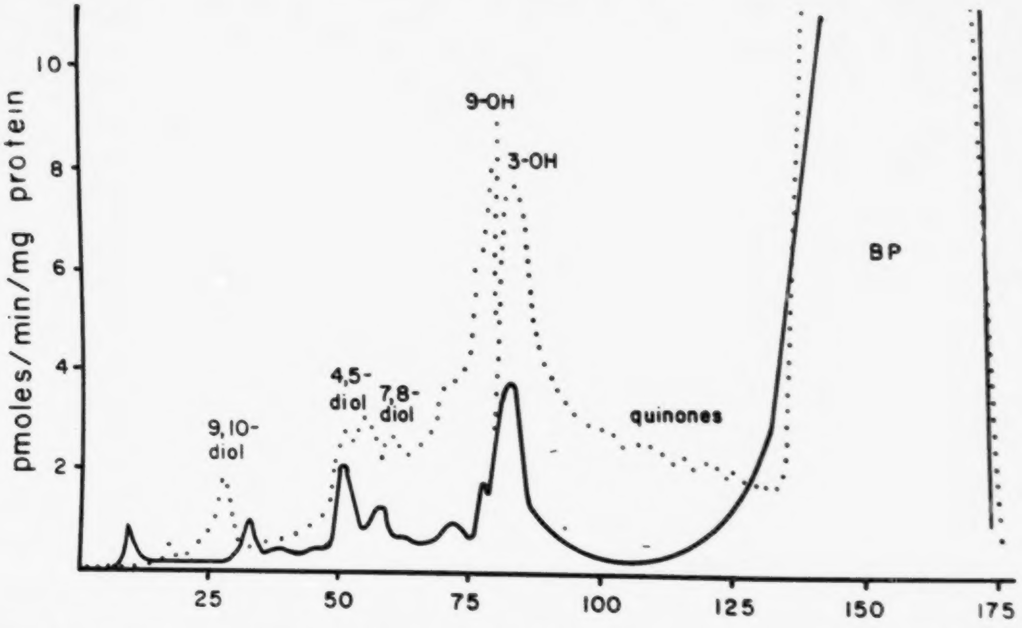

FIGURE 24. Effect of BF addition on distribution of radioactivity in HPLC eluate folowing incubation of ^{3}H-BP with liver microsomal fraction from control and 3-MC-pretreated channel catfish. Dotted line represents control plus _in_ _vitro_ BF. Solid line represents 3-MC plus _in_ _vitro_ BF.

Catfish liver microsomes metabolized BP into 9,10-diol BP, 4,5-diol BP, 7,8-diol BP, 9-OH BP, 3-OH BP, and quinones. Similar findings have been reported (83,128) using HPLC to study BP metabolism by control trout liver microsomes. Untreated trout liver microsomes metabolized BP into diols, hydroxy derivatives, and quinones in quantities exceeding those produced by control rat liver microsomes.

Several similarities between the rat and the catfish systems have been observed as far as BP metabolite profiles are concerned. The differences include the shoulder prior to the 7,8-diol BP, the lack of three resolvable quinone peaks as compared to the rat and multiple hydroxy peaks in the 3MC-pretreated fish.

Inter-tissue and inter-species differences in metabolite profiles exist. Selkirk et al. (49,129) have studied species differences in BP metabolism. The rat liver microsomal metabolite pattern was referred to as the "normal" profile and was used as a basis for comparison. The pattern from mouse liver microsomes is different in that much more 9-OH BP is formed than in the rat, and only one quinone peak is resolved (as found in the catfish). HPLC patterns for hamster liver microsomes show a marked decrease in 9-OH BP, relatively low 9,10-diol BP and 7,8-diol BP, but extremely high 4,5-diol BP.

Patterns for human liver show many peaks not seen in rat liver chromatographs. Human liver has two more peaks in the diol region, one after

9,10-diol BP and a small peak after 7,8-diol BP, a few additional quinone peaks, and one peak prior to BP. The goal is eventually to establish the relationship of metabolism to carcinogenesis by examining metabolite patterns for susceptible and refractory strains and species.

Pretreatment of catfish with 3-MC and PCB, and trout with 3-MC, significantly enhanced the metabolism of BP into 9,10-diol BP and 7,8-diol BP. The total BP metabolized in PCB-pretreated catfish (3%) was lower than the range for controls (14-22%). A great deal of variation was seen in BP metabolism among fish; however, treated fish generally metabolized more BP. Some 9,10-diol BP and 7,8-diol BP formation may be at the expense of 4,5-diol BP, which decreased in 3-MC-pretreated trout, PCB-pretreated catfish, and one 3-MC-pretreated catfish. This study has demonstrated that in vivo exposure of catfish and trout to xenobiotics affects the in vitro metabolism of BP.

BF reduced metabolism of BP significantly in samples from 3-MC-pretreated fish and enhanced metabolism in control samples (Figure 24). This correlates well with characterization in which AHH was enhanced in control samples by addition of 0.1 mM BF. AHH, however, was inhibited in microsomal fractions from 3-MC-pretreated fish after the in vitro addition of 0.1 mM BF. Selkirk et al. (130) also found decreased metabolism by 3-MC-pretreated rat liver microsomes with in vitro addition of 0.1 mM BF. Since the formation of every metabolite is affected, the inhibition must take place at the oxidase level (119).

BP is metabolized by rat liver microsomes to trans-BP-7,8-diol, which has been reported to bind readily to DNA of cultured human bronchi (131). Further metabolism produces a metabolite more mutagenic (132,133), BP-7,8-diol-9,10-epoxide. This diol epoxide of BP-7,8-diol causes the major damage to DNA in cell cultures (133) and in mouse skin (134). Yang et al. (131) have reported this diol epoxide to be produced by cultured human bronchi with subsequent binding to DNA.

Evidence has been reported that this mechanism is similar in trout (128). The metabolite BP-7,8-diol, the precursor of BP-7,8-diol-9,10-epoxide, was found to be quite predominant in trout by HPLC. This study (128) also used Sephadex column chromatography to resolve several BP metabolite-nucleoside complexes. The most predominant in trout and rat were BP-7,8-diol-9,10-epoxide and a peak associated with BP-phenols. Trout liver microsomes were also able to catalyze the binding of BP metabolites to DNA at a rate 3-4 times higher than rat liver microsomes. Although in vitro binding to DNA cannot be correlated directly to in vivo binding or biological effects, it has been shown that reactive intermediates formed by trout liver microsomes can produce mutations in Salmonella typhirmurium (135,136).

With the channel catfish, most of the metabolites formed seem to be identical to those formed by rat liver microsomes. The major differences noted are two peaks in BP-7,8-diol region and multiple peaks in the hydroxy region. Although three quinones can be resolved in the rat, only one quinone peak is seen in the catfish.

Species differences in BP metabolism have also been noted between rat and human tissue (137). On the basis of the data presented here, it can be deduced that fish liver microsomal systems can catalyze the metabolic formation of an ultimate carcinogen from BP *in vitro*. As was discussed in the introduction, only 1 report exists of tumor production in fish by laboratory exposure to BP (24).

The microsomal work done so far in fish must be followed by studies on whole cell systems. Levels of reactive intermediates during *in vivo* metabolism of PAH depend on the balance between activating and degrading enzymes and cellular nucleophiles like glutathione (137). Whole cell studies approach the *in vivo* metabolism of BP more closely than do microsomal studies.

Microbial Mutagen Assays

The results of the microbial mutagen assay using S-9 from fish with the four tester strains of *Salmonella typhimurium* and AAF, BP and MNNG are given (Table 10). AAF and BP, when incubated with S-9 and cofactors of the MFOS, were positive in all four tester strains. MNNG showed a variable effect (i.e., positive in TA-100 and TA-1535 but negative in TA-98 and minimal in TA-1537).

TABLE 10. INDUCTION OF MUTATIONS USING SALMONELLA TYPHIMURIUM STRAINS AND S-9 FRACTION FROM CATFISH LIVER

Tester Strain	Compound (a)	Revertants/ug CMPD/mg Protein (b)
TA-98	AAF	72.7±21.5
	BP	155.1±85.8
	MNNG	No response
TA-100	AAF	110.7±36.1
	BP	112.0±68.0
	MNNG	43.2+8.3
TA-1535	AAF	86.0±38.0
	BP	68.5±22.3
	MNNG	56.6+6.8
TA-1537	AAF	41.8±14.8
	BP	32.9±13.3
	MNNG	1.3+0.4

(a) Compounds: AAF (2-acetylaminofluorene); BP (Benzo(a)pyrene); MNNG (N-methyl-N'-nitro-N-nitrosoguanidine).

(b) Five concentrations of S-9 protein (0.17 - 2.51 mg/plate) used per each of 6 concentrations (5-30 ug/plate) test compound.

Values represent the number of revertants per ug of compound tested per mg protein ± S.D. subsequent to the subtraction of the number of spontaneous revertants (background level).

The effect of fish protein (S-9 concentration) upon the number of revertant colonies observed when concentration of test compound is kept constant (20 ug/plate) is shown (Fig. 25).

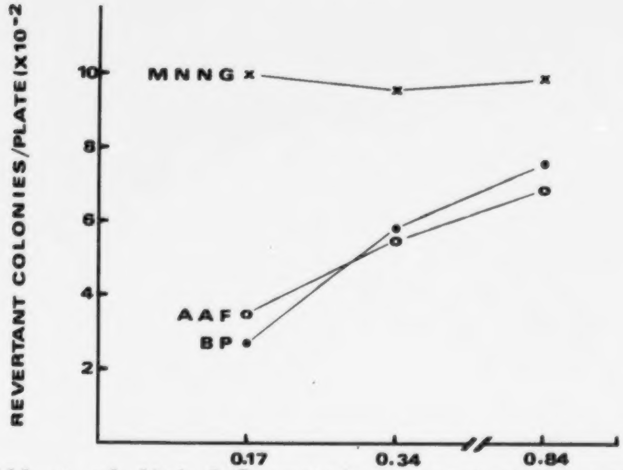

FIGURE 25. Effect of fish S-9 protein concentration upon the number of revertant colonies (TA-1535). AAF = 2-acetylaminofluorene 20 ug/plate; BP = benzo(a)pyrene 20 ug/plate; MNNG = N-methyl-N'-nitro-N-nitrosoguanidine 20 ug/plate.

When AAF and BP were used, increases in S-9 concentration were associated with increases in numbers of revertant colonies. This response was nearly linear between S-9 protein concentrations of 0.17 and 0.35 mg/plate but flattened at higher protein concentrations. By contrast, changes in fish S-9 protein did not affect the number of MNNG-induced revertants. This compound does not require microsomal activation to act as a mutagen in the Ames assay (58, 103).

The microbial mutagen assay results show that postmitochondrial supernatant (S-9) from channel catfish liver converted AAF and BP into mutation-causing metabolites. These results correlate well with those obtained using S-9 from Finnish lake trout (135) with AAF, BP, and aflatoxin. In the present study, test compound, S-9 and bacterial tester strains were incubated simultaneously for the customary (57) 48 hr period at 37°C. Since approximately 30% of catfish aryl hydrocarbon (benzo(a)-pyrene) hydroxylase activity is lost at 37°C our numbers of revertant colonies may be somewhat lower than those possible if a preincubation step (136) at temperature optimum for catfish MFOS had been used. However, the capacity of catfish liver S-9 to produce mutagenic metabolites of the above compounds has been established.

Carcinogen Interaction with In Vitro Organ Explant and Cell Cultures

The original purpose of this phase of the project was to test the ability of fish cells and tissue to convert a known procarcinogen to active metabolites capable of binding to host DNA of fish. The binding of carcinogen metabolites to cellular DNA is prerequisite for expression of carcinogenesis (104). Work in our laboratory performed in conjunction with the Human Tussue Study Section of the National Cancer Institute has shown that BP metabolites bind to human bronchi, colon and pancreatic duct DNA in organ culture.

Organ culture involves the maintenance of tissues in an *in vitro* environment such that morphological and biochemical characteristics of the *in vivo* organ are maintained. In recent years, attempts to culture a variety of mammalian and amphibian organs have met with good success. Organ culture has proven to be particularly valuable in mutagenesis/carcinogenesis studies in that it has allowed for the direct comparison of the human and animal response to a carcinogen. It is, therefore, surprising that relatively few attempts have been reported for the culture of teleost organs.

Since methods for organ explant culture of fish organs have heretofore not been described, it was necessary that we develop these prior to *in vitro* binding experiments. Accordingly, we have developed methods that allow for morphologic maintenance of several fish organs *in vitro* for up to 2 months duration.

Attempts were made to culture explants of gill, liver, skin, and kidney from channel fish. Skin and kidney were maintained *in vitro* for periods up to 2 months with little morphological change from tissue observed *in vivo*. Gill and liver provided inconsistent results despite considerable experimental manipulation of culture medium, temperature, and oxygen concentration.

Catfish skin was morphologically sampled immediately after being put into culture. Stratified epidermis contained epithelial, mucous, and alarm cells elements. Immediately basal to the epidermis, is the thick connective tissue of the dermis which is bordered on the underlying side by musculature.

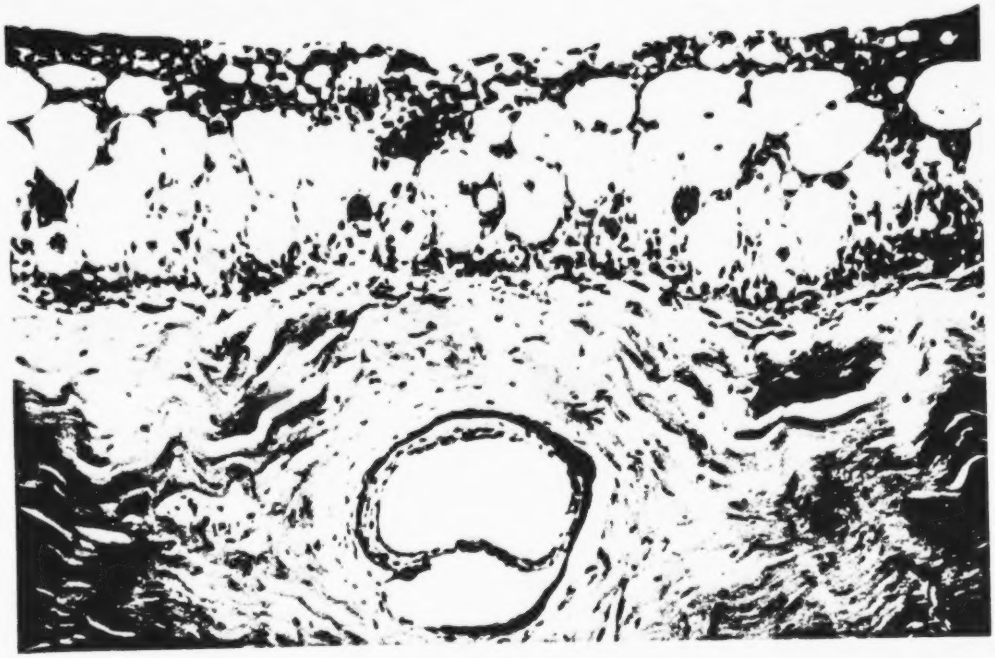

FIGURE 26. Light micrograph of channel catfish skin sampled immediately after being placed in culture. X 200.

After one week in culture, the epidermal layer can be seen growing down and around the cut surface of the explant eventually infiltrating the underlying dermis (Figure 27).

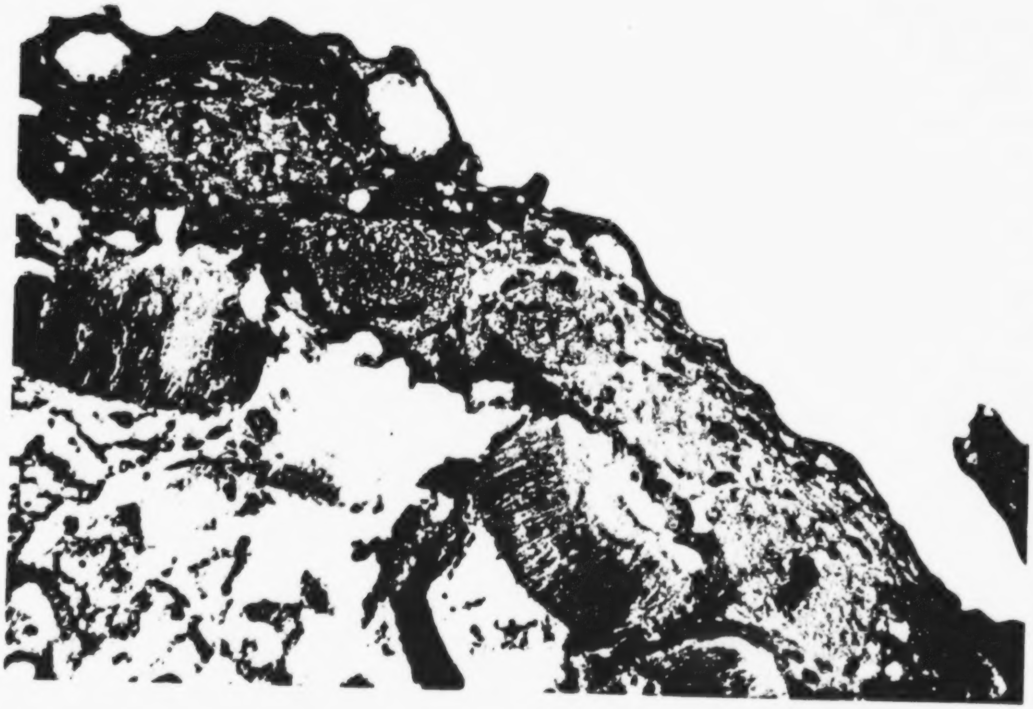

FIGURE 27. Light micrograph of channel catfish skin after one week in culture. X 300.

When the skin was examined after two months of culture, the epidermis was preserved in a fashion resembling morphology at 0 time (Figure 28).

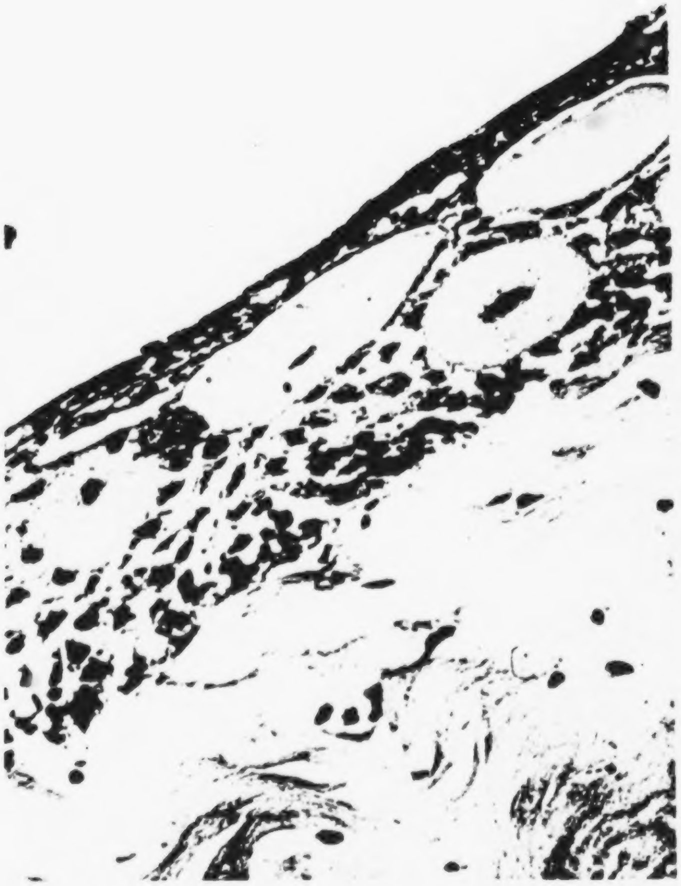

FIGURE 28. Light micrograph of channel catfish skin in culture for 8 weeks. X 200.

The kidney at 0-time (Figure 29) showed numerous tubular profiles and glomeruli. Hematopoietic tissue elements filled the interstitial spaces.

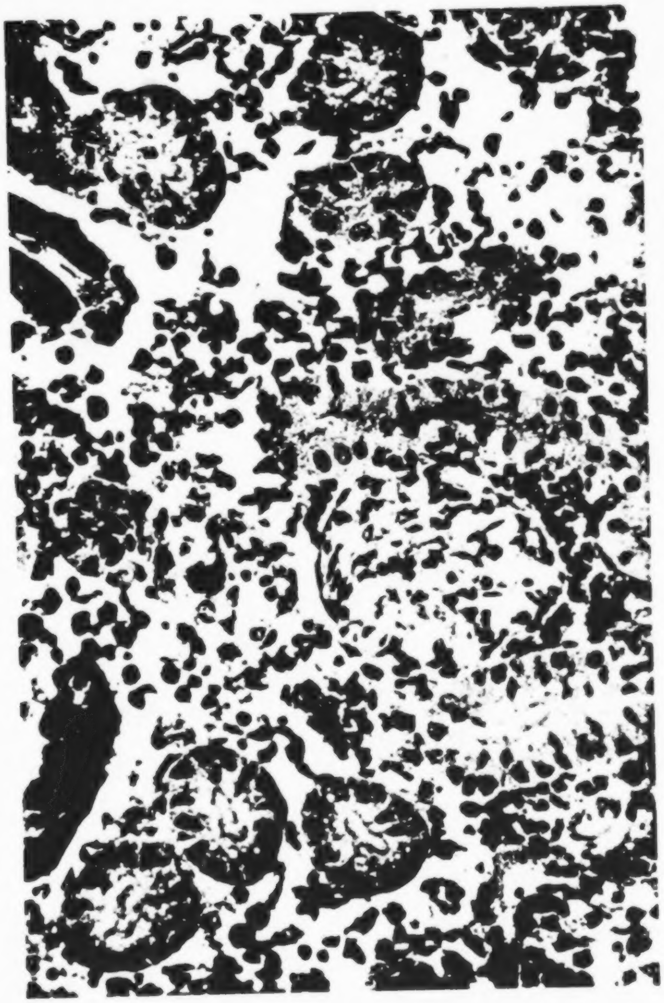

FIGURE 29. Light micrograph of channel catfish kidney at 0-time of culture. X 200.

After one week in culture, the tubule morphology remained well-maintained (Fig. 30). Glomeruli appear to be preserved while the numerous blood forming elements of the tissue have virtually disappeared.

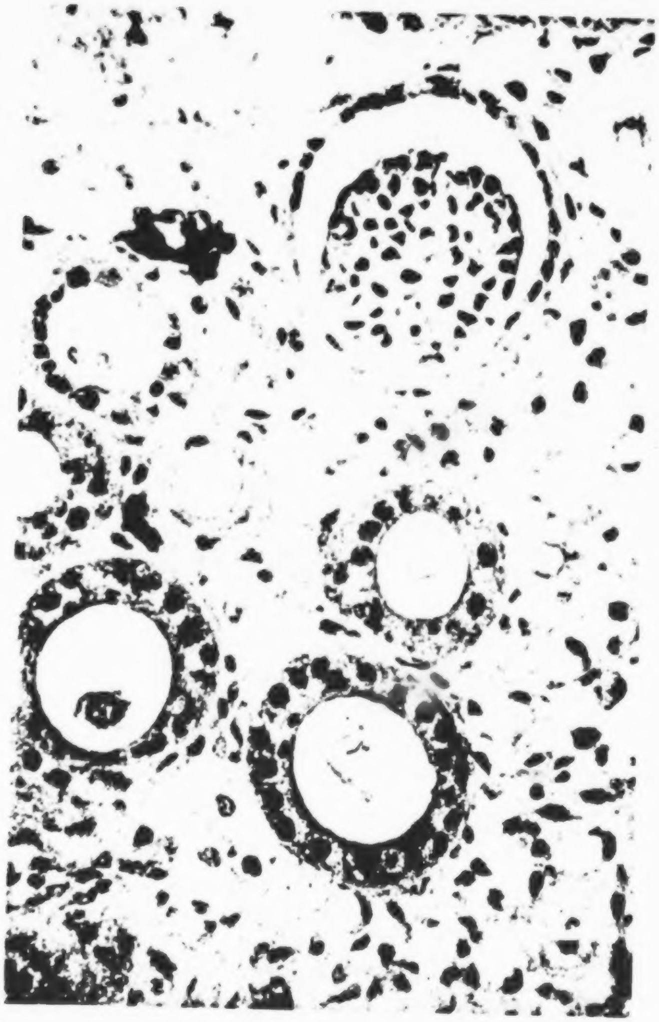

FIGURE 30. Light micrograph of channel catfish kidney after one week of culture. X 300.

Normal appearing tubules are still visible after two months culture (Fig. 31). The interstitium of hematopoietic tissue has been replaced by fibrous tissue. The parietal and visceral layers of Bowman's capsule are still maintained, thus preserving glomerular structure.

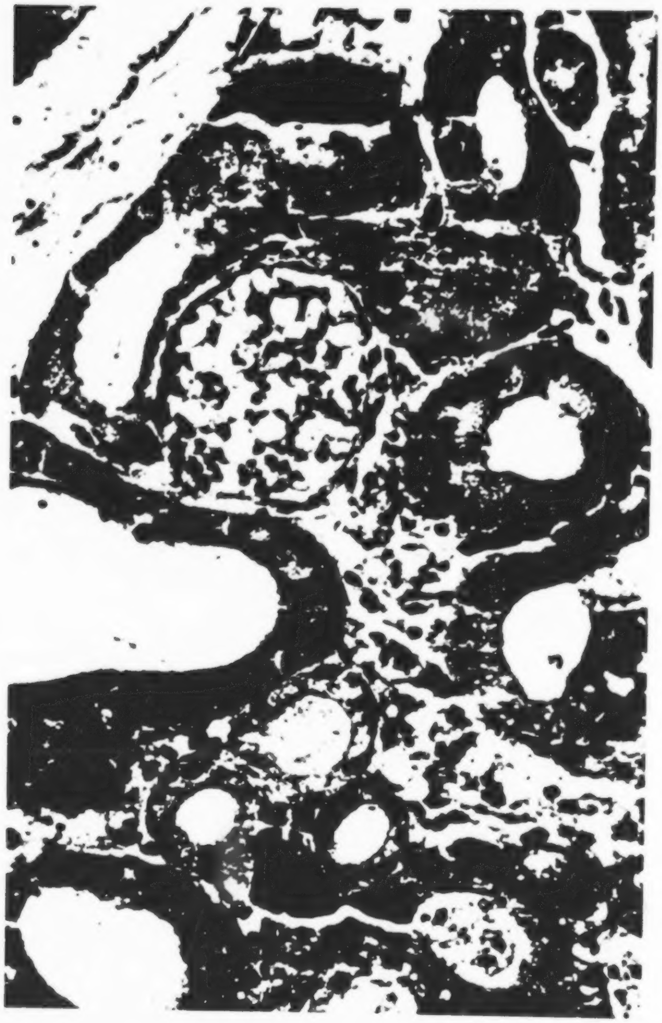

FIGURE 31. Channel catfish kidney in culture for 8 weeks. X 300.

The liver at 0-time is shown (Figure 32). A portion of a hepatic lobule displays a central vein surrounded by hepatic parenchymal cells in a dual plated pattern. The lobule is demarcated by portal triads containing hepatic veins, hepatic arteries, portal veins, and exocrine pancreatic tissue.

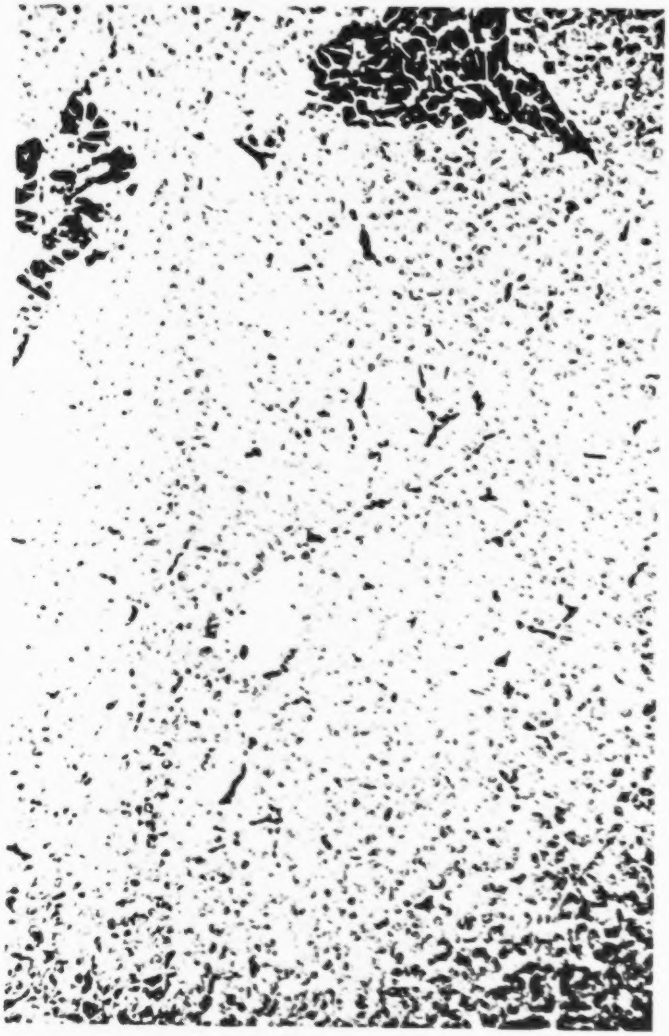

FIGURE 32. Portion of channel catfish liver sampled immediately after placement in culture. X 200.

Following two days in culture, hepatocytes and exocrine pancreatic cells show necrotic change (Figure 33). Some of the stromal and sinusoidal lining elements appear viable but these cells are in the minority.

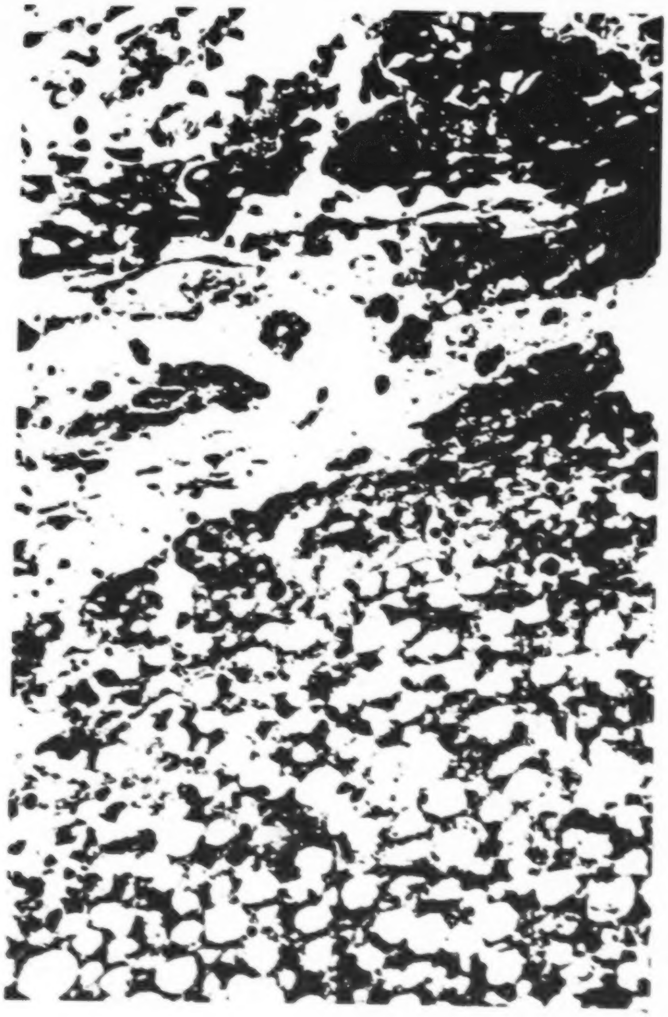

FIGURE 33. Liver after two days in organ culture. X 300.

Our ability to maintain kidney and skin in long-term culture may prove useful in in vitro carcinogenesis, mutagenesis, and toxicity testing. Similar organ explants from mammalian species have been maintained and these may prove useful in comparative studies. Although kidney and skin explant cultures proved successful, the results obtained with liver were disappointing. The teleost liver is the major site of experimentally-induced tumors (see Table 1) and as such would provide an ideal subject for in vitro assay. The liver also possesses an active and inducible MFOS and has been shown to be sensitive to a broad spectrum of procarcinogens. The epithelial nature of the liver also qualifies it as a model for epithelial carcinogenesis in general. For these reasons, we attempted culture of isolated liver cells.

Optimal methods for isolation of the catfish liver cells were developed. Liver pieces were trypsinizied at 25°C for variable periods of time. The results of this study are shown in Table 11. Eight hours trypsinization gave the greatest yield of viable liver cells as established via trypan blue staining and direct counting with a hemocytometer. The yield of viable cells was 3.25×10^6 per gram body weight (2.8×10^6/g liver). An average viability (trypan blue exclusion) of 98% was obtained.

TABLE 11. EFFECT OF DURATION OF TRYPSINIZATION ON CATFISH LIVER CELL YIELD

Duration	Total # of Cells Isolated	% Viable	Total # of Cells Per g bw	Total # of Viable Cells Per g bw
2 hrs	36.8×10^6	96%	0.49×10^6	0.47×10^6
4 hrs	98.3×10^6	99%	1.31×10^6	1.30×10^6
8 hrs	243.5×10^6	98%	3.25×10^6	3.18×10^6
12 hrs	7.3×10^6	75%	0.08×10^6	0.06×10^6

Temperature of trypsinization was 25°C.

Following isolation, liver cells were plated on to 90 mm^2 culture dishes and allowed to stabilize for 24 hrs. Representative plates were sampled for viability by phase contrast microscopy and trypan blue staining after 5 and 10 days. Cells were also sampled for electron microscopy. Figure 34 shows a portion of the isolated pellet viewed under the light microscope. A similar region, processed for electron microscopy is shown (Figure 35).

The ultrastructural characteristics of the isolated liver cells were indistinguishable from those of the intact liver. After 5 (Figure 36) and 10 (Figure 37) days of culture increases in glycogen content were noted. Cells during these periods began to aggregate and junctional complexes were frequently noted.

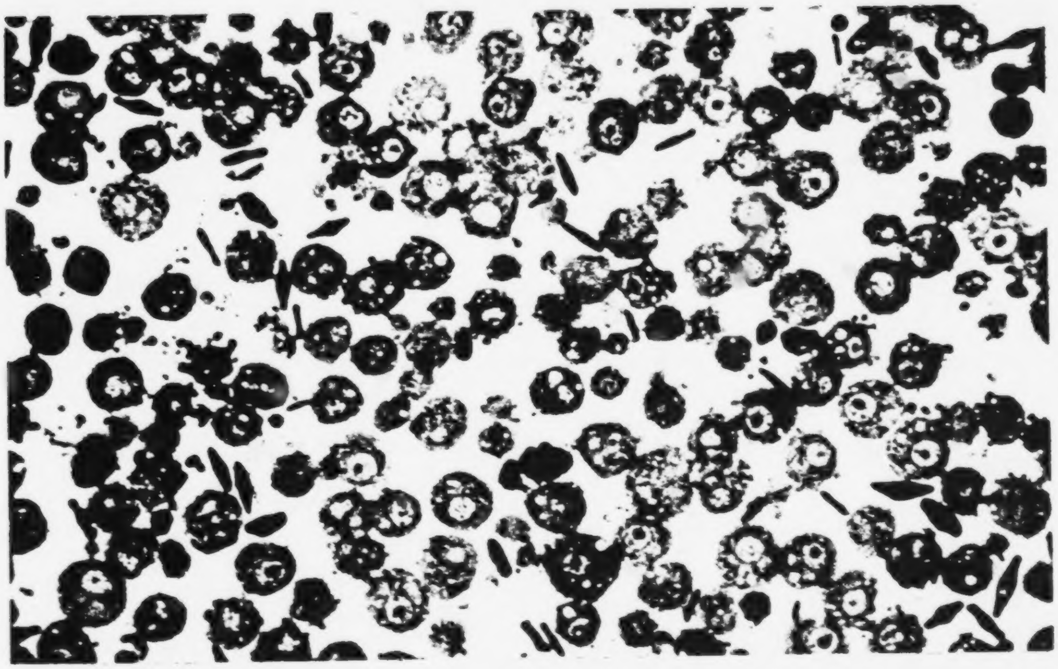

FIGURE 34 Light micrograph of channel catfish liver cell perfusion isolate. X 200.

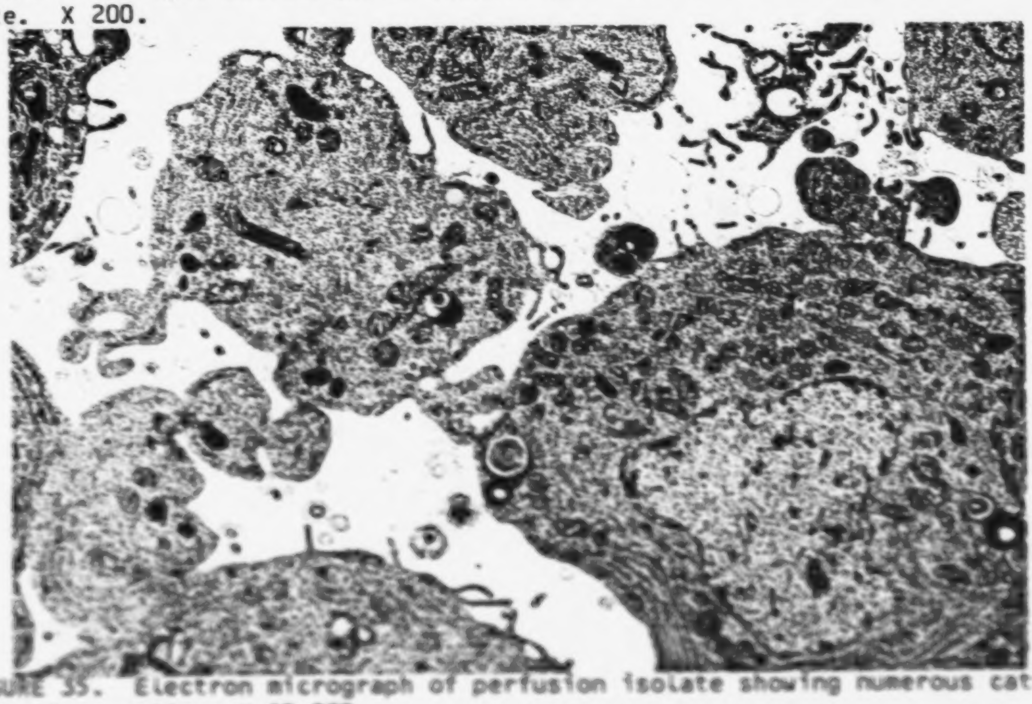

FIGURE 35. Electron micrograph of perfusion isolate showing numerous catfish liver cells. X 10,000.

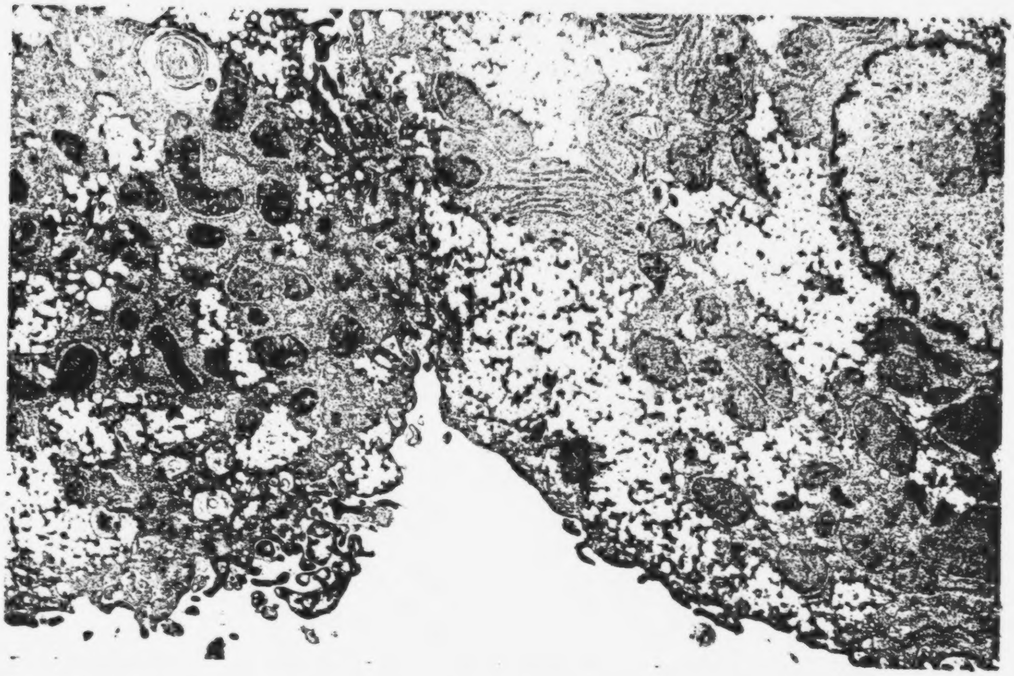

FIGURE 36. Electron micrograph of two hepatocytes after 5 days of culture. X 15,000.

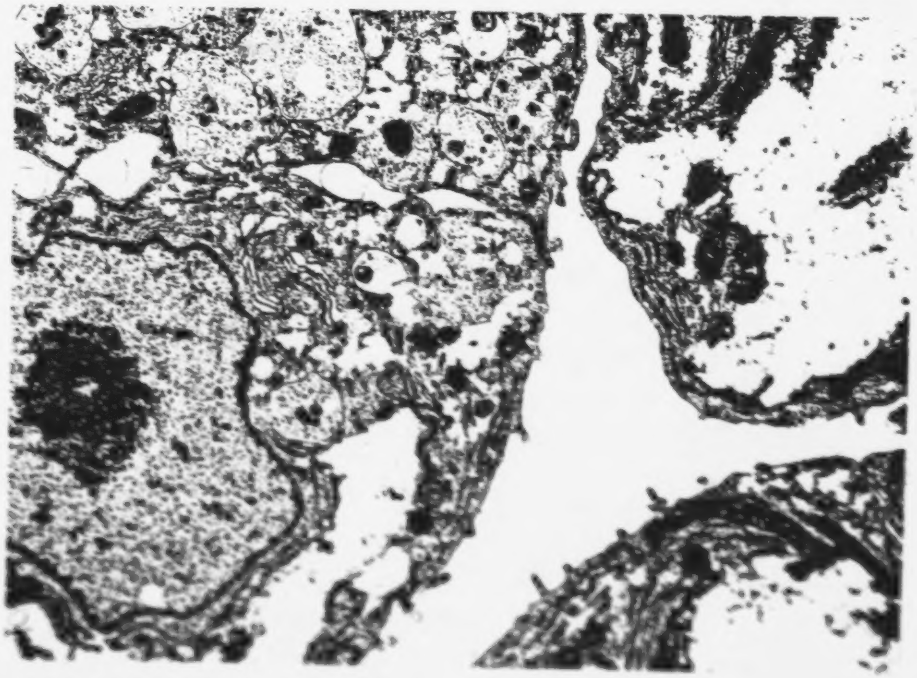

FIGURE 37. Several catfish hepatocytes after 10 days of culture. X 15,000.

Figure 38 is a photograph of a typical autoradiogram showing the localization of reduced silver halide grains over isolated hepatocytes following a 24 hr exposure to ^{3}H-BP. Grains were concentrated over nuclei (Table 12). Net nuclear grains (nuclear number less cytoplasm and background) averaged 10 (Table 12). Radioactivity of DNA isolated from liver cells following 24 hrs incubation with ^{3}H-BP averaged 490 dpm/ug.

TABLE 12. LOCALIZATION AND QUANTIFICATION OF ^{3}H-BP IN SUBCELLULAR COMPONENTS OF CULTURED PRIMARY FISH LIVER CELLS

Experiment #	Autoradiographic Analysis (grain counts over equal areas)[a]			Liquid Scintillation (dpm/ug DNA)[b]
	Nuclei	Cytoplasm	Intercellular (space background)	
1	33.4±14.7	18.4±6.3	6.2±1.7	480
2	30.8±11.8	19.5±7.1	6.4±1.2	380
3	37.8±12.8	15.2±5.4	5.8±1.4	630

(a) 1 x 10^6 cells exposed to 1.25 ^{3}H-BP (32 uCi/ml of medium).
Grain counts = mean ± S.D. of 100 nuclei, 100 randomly-selected cytoplasmic and 100 randomly-selected intercellular spaces of equivalent area/experiment.
(b) 5 x 10^6 cells as above.
Each experiment was performed on a separate cellular isolate from individual channel catfish.

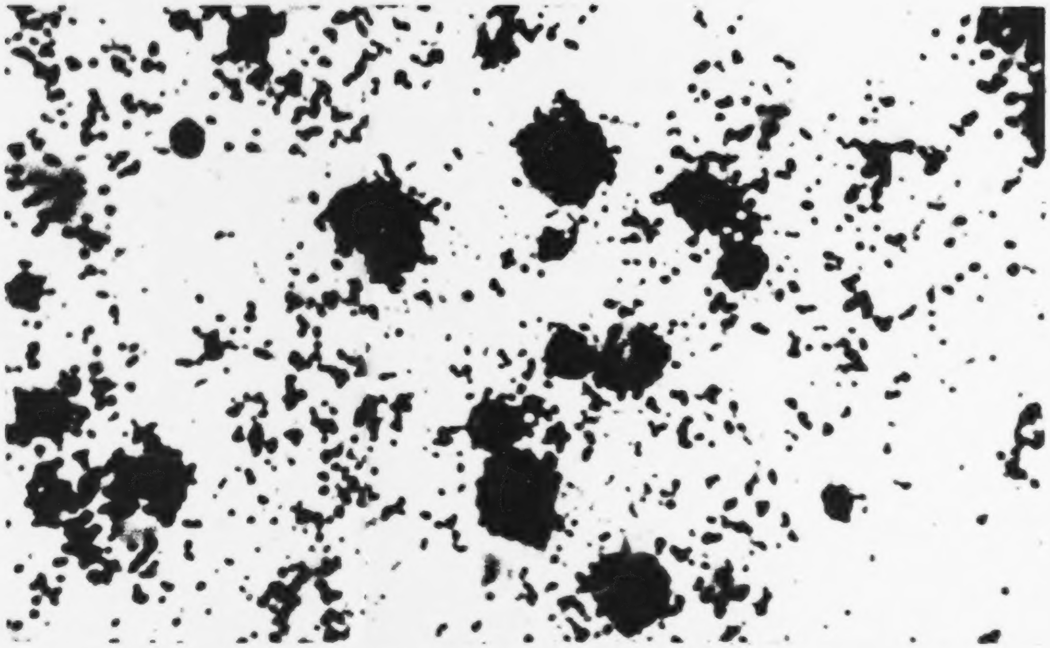

FIGURE 38. Autoradiograph of ^{3}H-BP treated catfish liver cells showing concentration of grains over nuclei. X 1,000.

One limitation of studies restricted to subcellular fractions is the occurrence of artifacts inherent in preparation which may damage key enzymes and/or disrupt compartmentation of others (138). The use of intact cells provides a system more akin to that of the whole organ or tissue from which the cell type of interest has been taken (139). In chronic laboratory exposures of fish to carcinogens, the most common target organ for tumors is the liver (see Table 1 introductory statement). For this reason, we focused major attention and time on the development of techniques for isolation and culture of primary fish liver cells. To date, the results have been most encouraging. High yields of viable cells have been routinely obtained. Initial experiments employing autoradiographic localization techniques after incubation of cultures with ^{3}H-BP (Figure 38) showed preferential localization over liver cell nuclei (Table 12). This finding, coupled with recovery of radioactivity in DNA fractions suggests interaction of BP with cellular components. Additional studies are needed to determine whether binding to cellular macromolecules has occured and, if so, to elucidate the nature of such binding. Since the metabolism of BP varies between species and between organs in the same species as well as with the type of preparation used (i.e., whole cells, microsomal fraction, or nuclear fractions) (49,140), further studies on metabolism, mutagenesis and in vitro carcinogenesis (141,142) using intact epithelial cells from fish are needed.

The geographic range of the channel catfish includes the entire eastern United States from southern Canada to Florida (143). In light of the projected increasing national reliance on coal-based energy production, regional contributions of environmental PAH from high point source emission associated with coal combustion, coking and conversion processes require surveillance of environmental quality (144). The response of the channel catfish to BP and 3-MC coupled with its ubiquitous occurrence in streams and lakes gives added importance to continued studies with this species including: varying routes of exposure, bioaccumulation, acute and chronic morphologic alterations and possible tumorigenesis. Such would help to determine the suitability of this species to serve as a sentinel organism for aquatic-borne PAH in fresh water habitats.

Morphologic Findings in Chronic Carcinogen Exposures

No gross observations suggestive of tumors were encountered in channel catfish exposed to AAF or to FBPA.

Findings in gills, skin, skeletal muscle, intestine, kidney, gonads, and liver of control channel catfish resembled closely the morphologic pattern described in previous accounts. Since major emphasis on the morphologic alterations following carcinogen exposure was placed upon liver, extensive analysis of this organ was performed. Control channel catfish liver of this study resembled very closely our previous description (113). Typical fields of liver at low magnification are shown (Figures 39-A,39-B). Exocrine pancreas comprising the hepatopancreas was found in adventitia of portal veins. Cords of cells existing as a dual-plated muralium were seen. Some regions of hepatocyte cytoplasm were opaque while other areas were nearly transparent in H&E stained preparations. The later corresponded to regions where glycogen was present. At later time periods (12 and 14 months) regions of control liver showed alterations in architectural pattern. In these areas (arrow Figures 39-A, 39-B) hepatocyte margins were indistinct and areas were interpreted as necrotic. In addition, control liver at 14 months showed occasional foci of round cell accumulation. These were interpreted as inflammatory foci and were comprised primarily of mononuclear cells. The above were located in portal and in some instances midzonal regions of hepatic lobules. High resolution light microscopic analysis (HRLM) (toluidine blue-stained sections of Epon-embedded material) revealed cytologic properties of control hepatocytes. In these, dark staining material was arranged as a perinuclear cuff with extensions to cell periphery. The hepatocytes contained one nucleus and were cuboidal to pyramidal in shape (Figure 39-C).

Randomly selected channel catfish were killed after 9 months of exposure to AAF and their tissues were processed for microscopic examination. Changes in tissues of exposed fish were compared to similar tissues in an equal number (9) of randomly selected control fish which were sacrificed at the same time. Only livers of treated fish showed morphologic alterations. Liver architectural pattern was unchanged. Focal sites of necrosis with vascular congestion were encountered in 8 of the 9 fish. Fat vacuoles were present in centrolobular and midzonal regions of the hepatic lobules (Figures 39-D). In two-thirds of the animals, peribiliary fibrosis was seen (Figure 40-A). This lesion was felt to be a later stage of a more commonly encountered pericholangitis seen in all exposed fish. A common finding in all fish chronically exposed to carcinogens was the biliary epithelial response. This included cytoplasmic vacuolization and pyknosis of nuclei in bile ductular and ductal epithelium (Figure 40-A). In addition, diffuse inflammation throughout the hepatic lobule was noted in one of the nine fish after 9 months of exposure

Twelve channel catfish were sampled after 12 months of exposure to AAF and their livers were compared to livers of 12 control fish maintained for a similar peroid of time. In all of the fish at this time, normal architectural pattern was found in livers. One out of twelve livers showed a hyperplastic focus (Figure 40-B). This lesion, smaller than one lobule in size, was detected as an area in which hepatocytes contained increased eosinophilia and basophilia and existed as trabeculae some 8-10 cells in thickness. Mitotic figures were seen in some hepatocytes (Figure 40-B) of the foci. Evidence of a chronic necrotizing process was seen in 7 of the 12 animals studied. Necrotic cells were seen throughout entire regions of the liver (Figure 40-C). In 3 of the 12 animals, necrotic areas were associated with localized vascular congestion. Two-thirds of the animals at this time showed evidence of peribiliary fibrosis (Figure 40-D) and 7 of the 12 animals showed diffuse inflammatory foci throughout liver lobes. Fatty change was encountered in a single animal at this time. Nonspecific tubular epithelial changes were seen in some kidneys. All other organs were nonremarkable. The number of fish surviving 14 months of exposure to AAF was 27. Morphologic alteration occurred in livers of all fish sampled at this time. Microscopic findings in other organs were nonremarkable. In general, hepatic architecture was maintained in all fish. Foci and areas of cellular alterations were encountered in 14 of the 27 fish. These lesions (see Figure 40-B) were typically comprised of cells which showed increased acidophilia and basophilia. They were larger than that seen at 12 months. In addition, these clumps of cells showed numerous mitotic figures indicating rapid growth characteristics. No tumors were encountered in any of the fish studied at this time. Hepatocyte nuclei in foci varied in shape and size. Some nuclei were enlarged and somewhat oval shaped. Others showed indentation and some appeared quite small. Most of the nuclei in foci of alteration were larger than nuclei of hepatocytes in adjacent tissues. A sharp transition existed between foci of alterations and surrounding hepatocytes although no compression was noted. Areas of necrosis were seen in the majority of the livers studied and frequently these areas were also sites of sinusoidal congestion. When present, fat droplets appeared as clear rounded spaces within hepatocytes sometimes existing as fatty cysts (Figure 41-A) associated with several nuclei. The cyst-like fatty lesions were focal and were seen in 6 of the 27 livers. Bile duct proliferation (Figure 40-D) was encountered rarely (in 3 of 27 animals). However, peribiliary fibrosis was a frequent finding (in 11 of 27 livers). PAS positive, diastase resistant material was abundant in cells lining sinusoids, in granules within hepatocytes (Figure 41-B) and in so-called melanomacrophage centers. This material apparently increased with duration of exposure and represents lysosomal (granules in hepatocytes) and phagocytic function of representative cell types. The increase of such material is interpreted as increased cell and organelle turnover due to the compound. Fish exposed for 14 months showed hepatocytes with rounded accumulation of acidophilic material in cytoplasm. This finding correlated well with the enhanced PAS positive, diastase resistant material.

The increased incidence of foci of alteration (hyperplastic foci) and areas seen at 14 months and their similarity to nodules described by others (21) in methylazoxy methanol acetate-treated fish, lead us to conclude that early lesions which may well have developed into overt neoplasia were occurring. However, the absence of distinct lesions causing compression of adjacent cells in non-involved regions makes this conjecture at this time.

Channel catfish exposed to FBPA for one week were studied by HRLM. Normal liver architectural pattern was seen in all 4 animals studied. Cytologic features were identical to that reported for controls above (Fig. 49). After 1 month of exposure to FBPA, five fish were taken for analysis by HRLM. In all of these, a normal liver architecture was seen. In one of the five animals, a small focus of necrotic hepatocytes was observed (Figure 41-C). Two of the five fish showed small foci of inflammatory cells in liver parenchyma. Cytologic features of individual hepatocytes was identical to that described in control. Four fish were killed after two months of exposure to FBPA and were processed for HRLM. In these individuals, normal liver architectural pattern was seen. In two of the four animals, small areas were observed in which cells showed a disorganized pattern of dark staining material and it was difficult to distinguish between individual cells in these areas. These foci were taken to represent necrosis (Figure 41-C). No fat was observed. Bile ductular proliferation was not seen. Peribiliary fibrosis occurred in 2 of the 4 animals studied. All other features were identical to controls. After 3 months of exposure to the compound, two animals were taken and compared to two controls maintained for a similar period of time. The only alterations observed in these livers were small sites of necrosis within treated fish similar to areas described above. Cytologic features of individual hepatocytes were identical to controls. After 6 months of exposure to FBPA, 3 treated and 3 control fish were taken for HRLM. In treated and control fish, a normal liver architectural pattern was seen. In one of the 3 treated fish, dark staining regions within cytoplasm were irregularly arranged; however, the response was focal. No necrosis, fat, bile duct proliferation, peribiliary fibrosis or inflammation was observed. Cytology of individual hepatocytes (Figure 41-D) revealed dispersal of dark staining material peripherally with central portion of cells containing light staining material. This pattern of cytoplasmic rearrangement is similar to that seen in hepatocytes in which smooth endoplasmic reticulum has proliferated (43). Livers from 6 treated and 6 control fish were studied by routine light microscopy after 9 months of exposure to FBPA. In general, liver architecture was well-maintained. However, in 2 of the 6 fish, areas in liver sections were seen in which sinusoids were not apparent. Cytoplasm of cells in these regions revealed a hyalinized appearance. Necrosis was not found in animals at this time. Fat was seen as clear vacuoles within hepatocytes in one of the 6 animals. In addition, the same liver showed diffuse small foci of inflammatory cells. After 12 months of exposure to FBPA, 5 of 6 livers observed showed normal architectural pattern. In the other, the outer margins of the liver revealed numerous bulges and alternating constrictions resulting in a "scalloped" appearance. Necrosis was seen in 2 of the 6 livers examined at this time. Sites of necrosis and pyknosis were common (Figure 42-A). In 3 of the 6 livers, areas were seen in which cytoplasm of hepatocytes revealed increased opacity (Figure 42-B). In addition, these areas were sites of vascular congestion. Fat was observed in 2 of the 6 animals as clear vacuoles within hepatocytes of centrolobular and midzonal regions. Bile duct proliferation, not seen in animals exposed for shorter periods of time to FBPA, now appeared in 4 of the 6 animals. In addition, peribiliary fibrosis was found in 4 of six animals. Bile stasis was indicated from the cut section appearance of ductules and ducts. These had expanded, perfectly rounded lumens and thin lining epithelial cells suggestive of increased intraluminal pressure. Five of the 6 animals studied showed inflammation particularly pericholangitis. Numerous mitotic figures were seen in bile ductular epithelium. In livers of 3 fish, bile ductal epithelial hyperplasia was apparent. One of these had progressed to papillary projections within the lumen. This configuration with numerous mitotic figures (Figure 42-C) is consistent with a diagnosis of cholangioma.

When compared to controls maintained for identical periods of time, livers of channel catfish exposed to FBPA for 14 months revealed changes. Both control and treated livers showed necrotic areas although these were by far greater in treated fish. Areas of necrosis in both groups were associated with fibrosis. Granulomas were seen in the liver of 1 control fish but not in treated fish. Hepatic architecture was similar in both groups. However, cytoplasm of hepatocytes from treated fish contained more stainable area-often completely filling the cell. Controls apparently contained abundant glycogen which, by H&E, did not stain, making these cells nearly transparent. Cytologic features of hepatocytes from the 14 month FBPA group included abundant acidophilic inclusions which were frequently as large as nuclei. In basophilic regions of cytoplasm, a fine vacuolization was seen. Nuclei, generally one per cell, were lucent, rounded and had a single prominent nucleolus (Figure 42-D). Treated livers revealed oval cell proliferation in 15 of 32 fish surviving the 14 month exposure. Peribiliary fibrosis, pericholangitis and melanomacrophage centers were common. In 8 of 32 livers, areas were seen in which cytoplasm appeared hyalinized and gave a general basophilic hue with H&E. Nuclei in these regions were more opaque and generally uniform. However, some nuclei were enlarged, oval in shape and indented. Cells in these regions existed as continuous sheets and it was difficult to visualize sinusoids. In one fish, the above described region was seen as a nodule which compressed adjacent hepatocytes. This led to our diagnosis of minimal deviation (115) hepatocellular carcinoma. Mitotic figures were seen in the above.

One half of the livers from this group revealed foci in which nuclei were literally piled one on top of another and mitotic figures were present These foci were termed hyperplastic. They were not associated with compression of adjacent hepatocytes. In some livers (4 of 32), extensive areas of necrosis were seen. In these regions, surviving hepatocytes stained darkly and appeared as nodular forms in acinar arrangement (Figure 43-A) or as thickened trabeculae (Figure 43-B).

Of 30 brown bullheads chronically exposed to 3-MC, 12 survived the 24 months exposure. Gross inspection of these fish revealed evidence of fin abrasion with small hemorrhagic areas near distal tips. These were more apparent on pectoral fins. Inspection of the liver surface revealed a total of 2 pale elevations of approximately .4 mm diameter (1 in each of 2 affected fish). All other internal organs were nonremarkable.

Microscopic examination of control bullhead tissues revealed typical teleost morphology. Detailed analysis of liver showed lobules with the typical dual-plated muralium of teleost liver. Abundant glycogen in hepatocytes gave the appearance of little stainable material in cytoplasm of H&E stained preperations. Exocrine pancreas was found in periadventitia of portal veins and near bile ducts and hepatic arterioles. Controls showed minimal deviation from normal liver morphology. These included small, infrequent foci of round cell accumulation and small sites of necrosis with fibrous connective tissue. A one micron section, stained by toluidine blue, through one of the pale elevations mentioned above, is shown (Figure 43-C). Apparently hepatocytomegaly and sinusoidal dilatation contributed to the gross findings In addition, the capsule appears thickened. When compared to control liver similarly processed (Figure 43-D), enlarged hepatocytes stained less intensely than did their control counterparts. H&E stained preparations of liver from 3-MC treated bullheads routinely showed yellow pigmentation over hepatocytes and foci of macrophage accumulations in which pigment was even more obvious. No tumors or nodules were seen in sections of treated liver. However, the liver from one fish showed 3 foci in which 5-8 hepatocyte nuclei were tightly aggregated. Evidence of mitoses was seen and cytoplasm adjacent to these nuclei was basophilic with respect to adjacent cells. Peribiliary fibrosis was commonly encountered.

Of 30 brown bullheads chronically exposed to BP, 26 survived the 24 month exposure. Examination of liver surfaces in these, with the aid of a dissecting microscope, revealed pale raised nodules (0.4 mm in diameter) in 5 of the 26 fish. One of these was subsequently found to be due to an encapsulated parasite of undetermined species. As in the 3-MC groups, sections of Epon-embedded material revealed hepatocytomegaly and margination of dark staining cytoplasmic constituents. No evidence of hepatocellular carcinoma was found. Similarly no evidence of altered foci was found. Changes included an enhanced pattern of trabeculae, sinusoidal dilatation (focal) with congestion, occasional necrotic and scarred areas, evidence of bile retention and increased incidence of melano-macrophage centers. Areas of hyalinized hepatocellular change was seen in one fish. In that area, fine vacuolation of cytoplasm was also noted. Histopathologic examination of gill, intestine, kidney and gonads revealed no changes. Interestingly, 2 exposed fish showed curvature of the spine. Both kyphosis and scoliosis were detected. Controls did not show this condition.

Of 50 fathead minnows at the start of the 24 month exposure to 3-MC, 7 survived the entire period. Analysis of our log for this study shows sporadic mortality separated by 7-20 day intervals in which no mortalities occured. When possible, all tissues from fish dying during the course of the experiment were processed for light microscopic examination. However, autolytic changes in many fish obviated our obtaining meaningful morphologic data. Based upon our analysis of moribund and dying fish, apparently exposure of fathead minnows to 3-MC for periods in excess of one month leads to hepatocytomegaly and margination of dark staining cytoplasmic constituents (Figure 44-A). Enlarged hepatocytes have large clear stained areas with dark staining components (mitochondria, lysosomes) restricted to small regions within cytoplasm. As discussed previously, we have linked this light microscopic finding to smooth endoplasmic reticulum proliferation in the rat (43). The same alterations have been shown by Schoor and Couch (145) in marine fish exposed to 3-MC. In one fish which was near death after 12 months of exposure and in another fish at the termination of the experiment, kyphosis and scoliosis were seen. Skeletal lesions in both fish were accompanied by protrusions from the lateral body wall. These were rounded elevations of approximately 1 cm maximal diameter. Sections through these lesions, stained by H&E are shown (Figures 44-B,C,D). At the center of the mass, an encapsulated cystic structure is shown which is connected to bone centrally. The periphery of the encapsulated mass is shown (Figure 44-C) Atrophic skeletal muscle is found adjacent to the capsule. Internally (Figure 44-D) the lesion is composed of collections of signet ring formations (fat?) separated by septal projections from the capsule. No other gross abnormalities were seen in the minnows examined at the termination of the exposure.

Four months into our exposure of the estuarine teleost, Fundulus heteroclitus to FBPA and to BP, an electrical outage caused compressor failure between the hours of 3:00 p.m. Saturday and 7:00 a.m. Sunday. At that time the majority of the fish in both exposure groups and the control tank were dead or dying. Autolytic change in livers prevented our analysis of all but 5 control fish, 5 BP-treated fish and 20 FBPA-treated fish. Gross inspection of the above was non-remarkable. The dual-plated muralium of the liver in this species is shown (Figure 45-A). Hepatopancreas was not abundant in this species. All sections from control fish revealed fat vacuolization ranging from a centrolobular location in some fish (Figure 45-B) to extensive vacuoles of various sizes throughout the lobule (Figure 45-C). Microscopic evidence of post mortem autolysis was seen in 3 of 5 control livers. No bile duct proliferation, peribiliary fibrosis or inflammation was seen in controls. Livers from BP-treated fish were not different in appearance from control morphology except for post mortem autolytic change in one of 5 fish. In the FBPA-treated fish, livers revealed a normal architectural pattern. Granulomatous reactions were seen in 6 of 20 fish livers. When small (Figure 45-D) these lesions resembled foci of hepatocellular alteration.

However, macrophages and mononuclear white blood cells were present particularly in larger lesions (Figure 46-A). Lining cells of hepatic sinusoids were frequently engorged with an acidophilic homogeneous material (Figure 46-B). Adjacent to some granulomatous lesions were foci of altered hepatocytes. These showed intense basophilia (Figure 46-C).

At the midpoint of this project, our group elected to add a bottom feeding species to the long-term exposure study. Since koi were readily available, exposure and control aquaria were assembled for FBPA feeding experiments. Interim analysis of koi exposed to FBPA for 3 months revealed no change in H&E stained liver sections. However, HRLM analysis showed cytomegaly and peripheral relocation of dark staining material. After 9 months exposure, seven control and seven surviving FBPA-treated fish were studied. Fatty change (cyst formation) was apparent in 2 of 7 exposed fish. In one fish, small foci of necrosis were seen. Additionally, pericholangitis and bile statis was seen in one fish. No gross or microscopic changes were encountered in other organs.

In the interpretation of liver lesions, we have consulted previous reports in various species (14-23,115). The response of the channel catfish liver to AAF and FBPA was encouraging. However, the latency period was long. In light of the amount of water required to maintain large numbers of this size fish and the problem of disposal of large amounts of contaminated water, use of smaller species such as small aquarium fishes (13) may be advisable. Further study of carcinogenesis in fish (see review by Sinnhuber et al. (1) could be extended to egg, embryo-larval exposures in which concentrated dosages of carcinogen are followed by rinsing and subsequent culture under routine conditions. These may be a feasible alternative to the above-noted problems. Egg, embryo-larval exposures need to be extended to other species in an effort to determine a ubiquitous, sensitive, indicator organism for aquatic carcinogen bioassay.

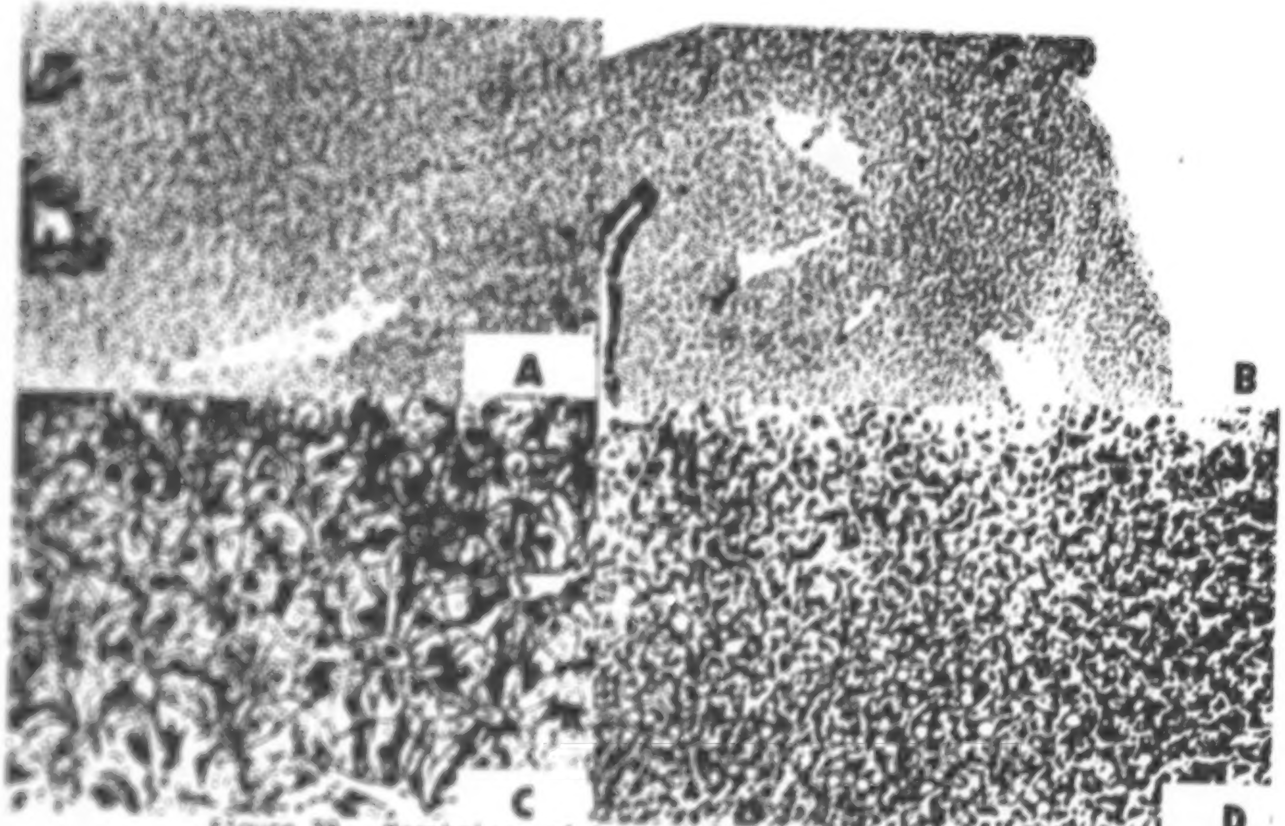

Figure 39. Morphology of Control and Treated Catfish Liver.

A. This field from control channel catfish liver is from central portion of section. Normal pattern of architecture is seen with lucent and opaque regions in hepatocyte cytoplasm. Exocrine pancreatic cells are shown in periadventitia of portal veins. Areas of necrotic change (right side, middle level) were encountered in controls of late time periods. Hematoxylin and eosin stain (H&E) X 450. B. Periphery of liver from control channel catfish shows a region of necrotic change at upper right. H&E X 450. C. Hepatocyte morphology in control channel catfish liver. Most cytoplasm is light staining due to glycogen accumulation. Dark staining material forms a perinuclear cuff with extension to the plasma membrane. Toluidine blue-stated Epon section. X 1000. D. Fat vacuolation in hepatocytes of midzonal region of hepatic lobule in channel catfish after 9 months exposure to AAF. H&E X 200.

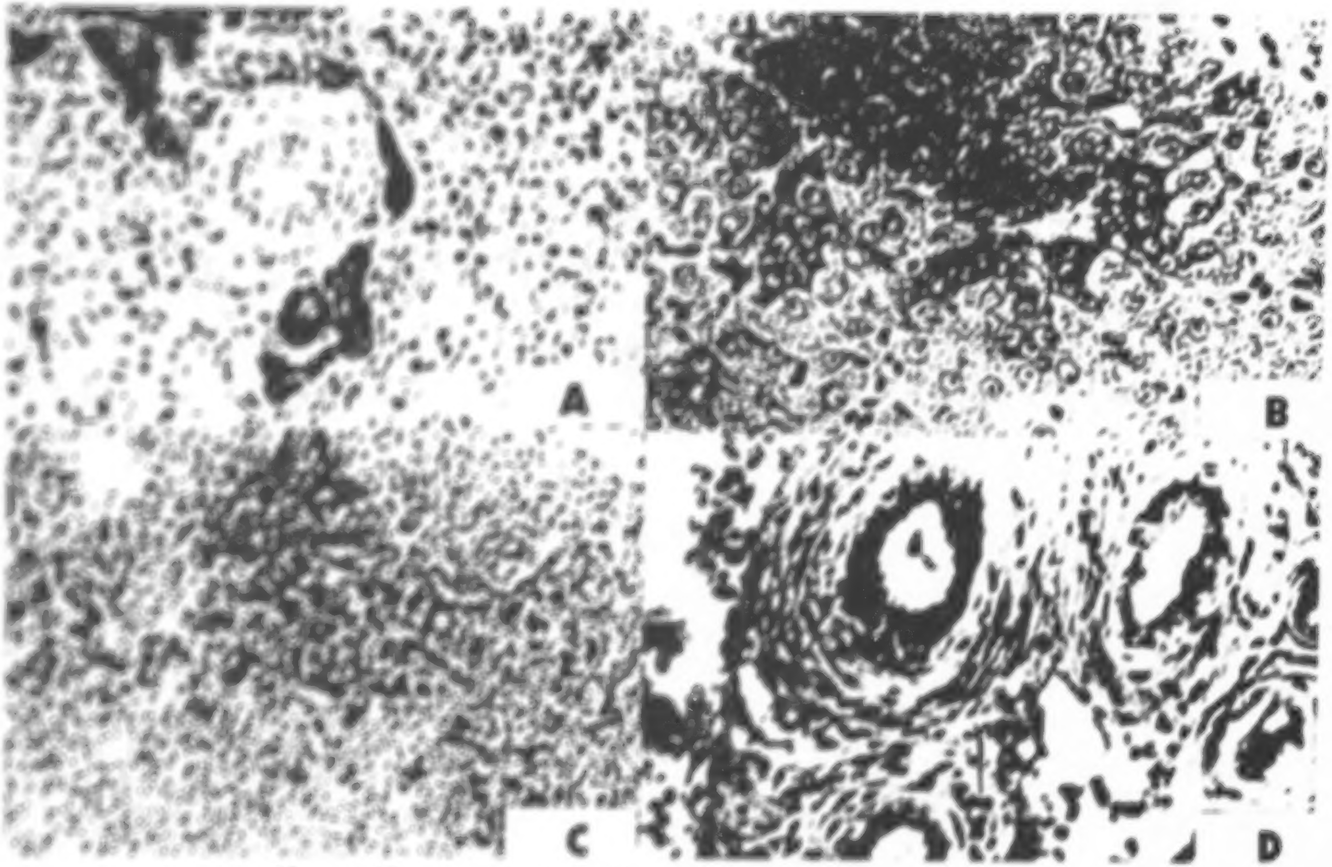

Figure 40. Lesions of Channel Catfish Liver.

A. Peribiliary fibrosis with epithelial nuclear pyknosis in a channel catfish after 9 months exposure to AAF. H&E X 200. B. Hyperplastic focus in channel catfish after 12 months exposure to AAF. Increased cytoplasmic staining and mitoses (arrows). H&E X 400. C. Large necrotic region with surviving hepatocytes stained darkly in a channel catfish liver after 14 months exposure to AAF. H&E X 200. D. Site of bile duct proliferation with fibrosis and mitotic figures in lining epithelium after 12 months of AAF. H&E X 400.

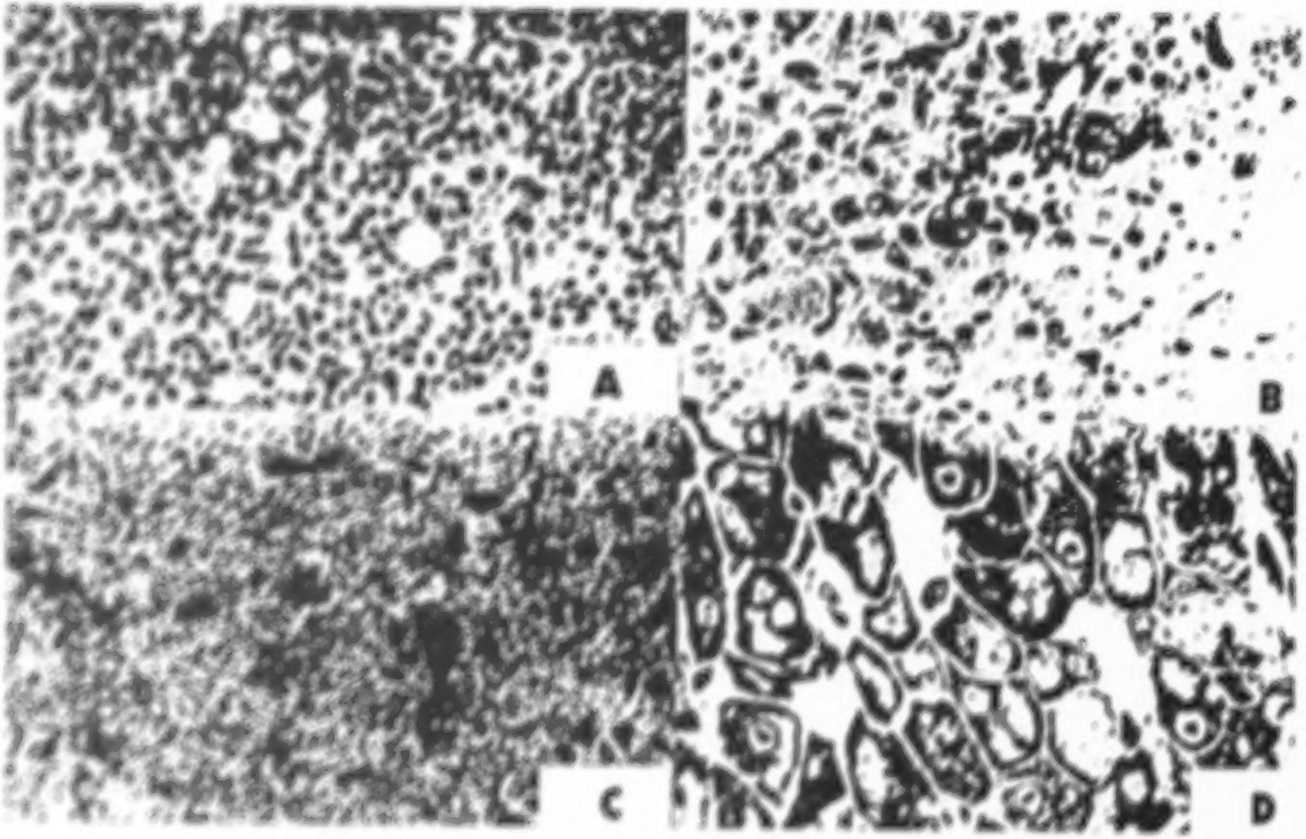

Figure 61. Morphologic Alteration in Channel Catfish Liver.

A. Fatty cysts in liver of channel catfish after 16 months exposure to AAF. H&E X 200. B. PAS - positive diastase-resistant material in melanomacrophage center and as granular deposits (arrows) in hepatocytes. PAS + diastase. X 200. C. Necrotic region in channel catfish exposed to FBPA for 1 month. Toluidine blue-stained Epon section. X 400. D. Enlarged hepatocytes with rounded margins and peripheral relocation of dark staining material. Toluidine blue-stained Epon section. X 1000.

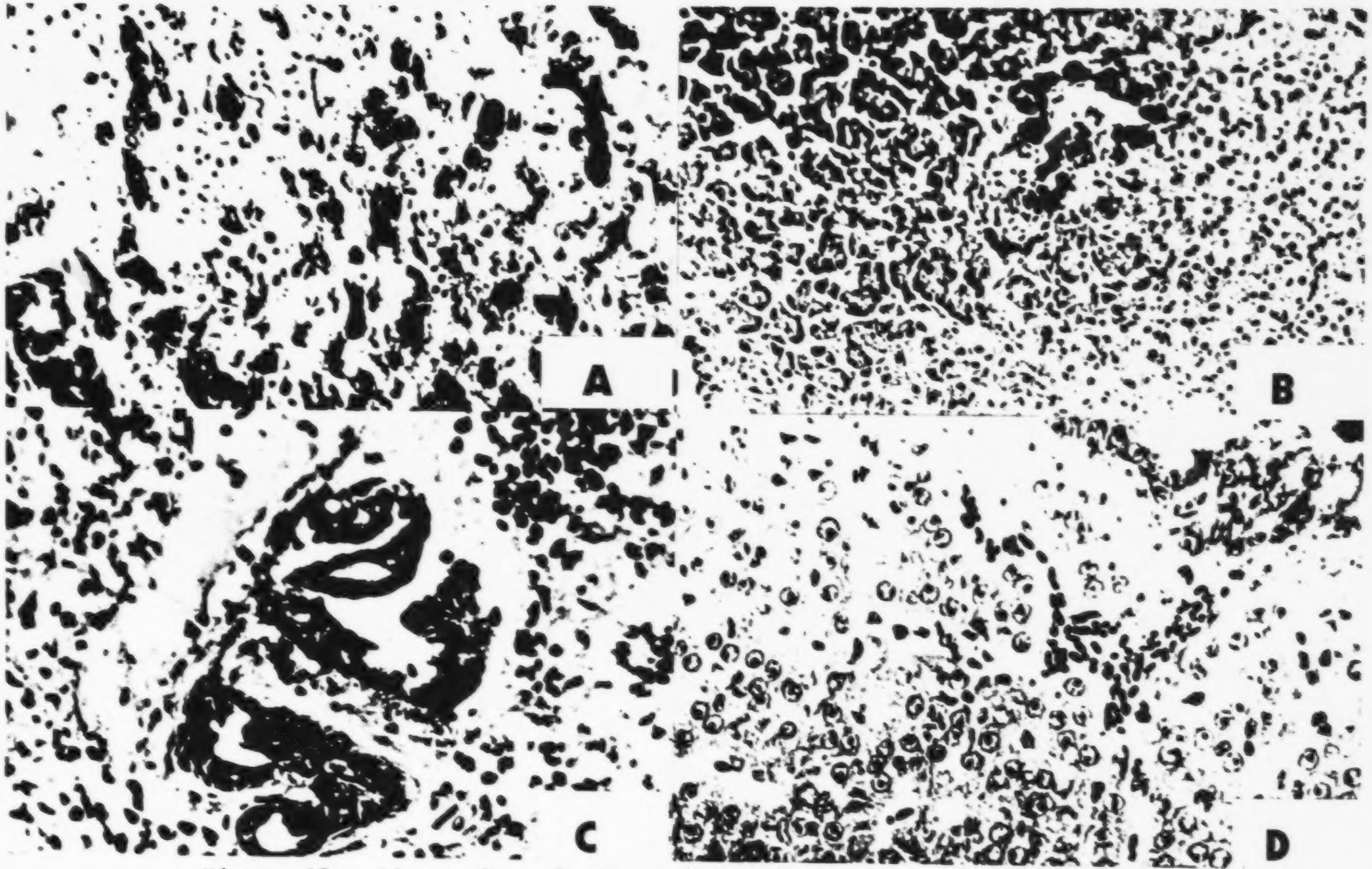

Figure 42. Alterations in Channel Catfish Liver after FBPA Exposure

A. Liver from channel catfish after exposure to FBPA for 12 months. Note necrosis and pyknosis of hepatocytes. H&E X 200. B. Liver from channel catfish after 12 months exposure to FBPA. Cells at right of field show more cytoplasmic opacity with a fine diameter vacuolization. Region above contrasts sharply with cells in acinar arrangement at left and center of field. H&E X 200. C. Cholangioma in channel catfish after 14 months exposure to FBPA. Papillary projections with numerous mitoses are seen. H&E X 400. D. Periportal hepatocytes in 14 month exposure to FBPA (channel catfish). H&E X 100.

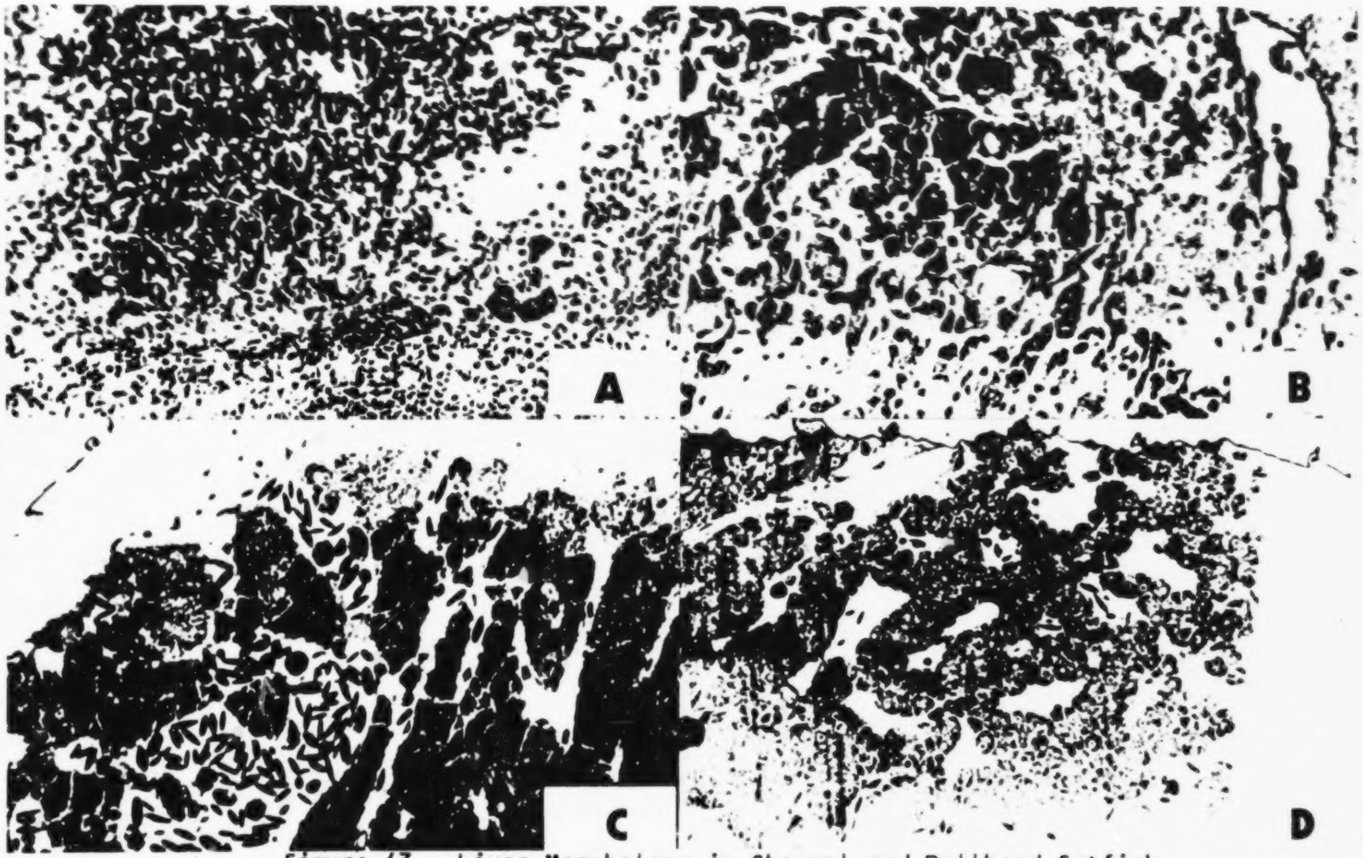

Figure 43. Liver Morphology in Channel and Bullhead Catfish.

A. Acinar nodule of hepatocytes in necrotic zone of channel catfish liver after 14 months exposure to FBPA. X 200. B. Thickened trabeculae of hepatocytes in necrotic zone of channel catfish liver after 14 months exposure to FBPA. X 400.

C. Brown bullhead liver after 24 months 3-MC exposure. Enlarged hepatocytes and focal sinusoidal dilatation are seen. Toluidine blue-stained Epon section. X 400. D. Liver cords in control brown bullhead. Toluidine blue-stained Epon section. X 400.

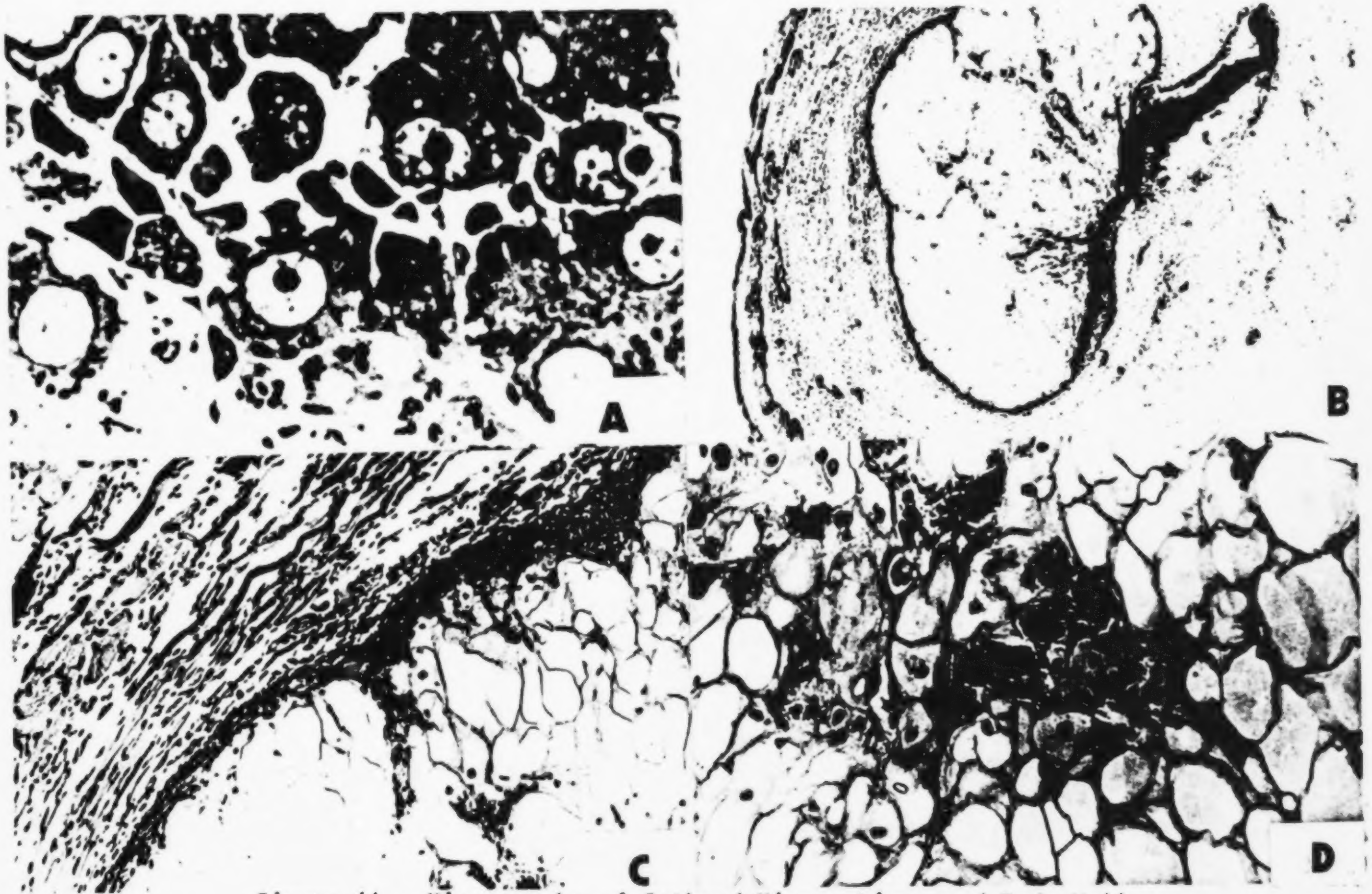

Figure 44. Micrographs of Fathead Minnow Liver and Body Wall.

A. Liver from fathead minnow after 3 months exposure to 3-MC. Hepatocytomegaly and focal aggregation of dark staining material are seen. Toluidine blue-stained Epon section. X 1000. B. Cystic lesion in body wall of fathead minnow exposed to 3-MC. H&E X 40. C. Periphery of cystic lesion shows compression of skeletal muscle. H&E X 200. D. Signet ring formation in central area of encapsulated cyst. H&E X 400.

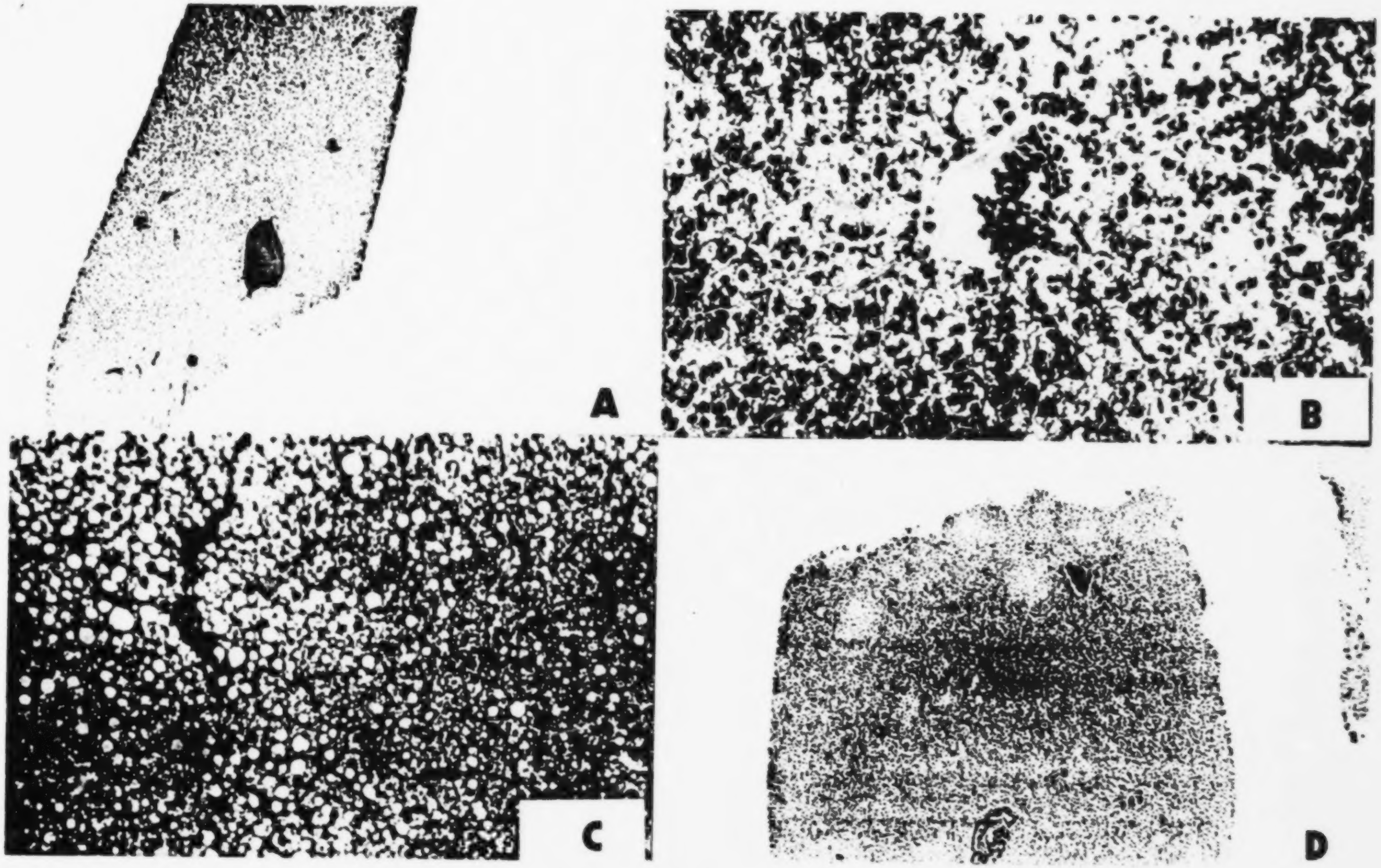

Figure 45. Micrographs of Liver From Fundulus Heteroclitus.

A. Control liver of Fundulus heteroclitus. H&E X 40. B. Fat vacuolization in centrilobular region of fundulus liver. H&E X 200. C. Maximum fat deposition in control liver of fundulus. H&E X 200. D. Multifocal granulomatous reaction in FBPA-treated fundulus liver. H&E X 40.

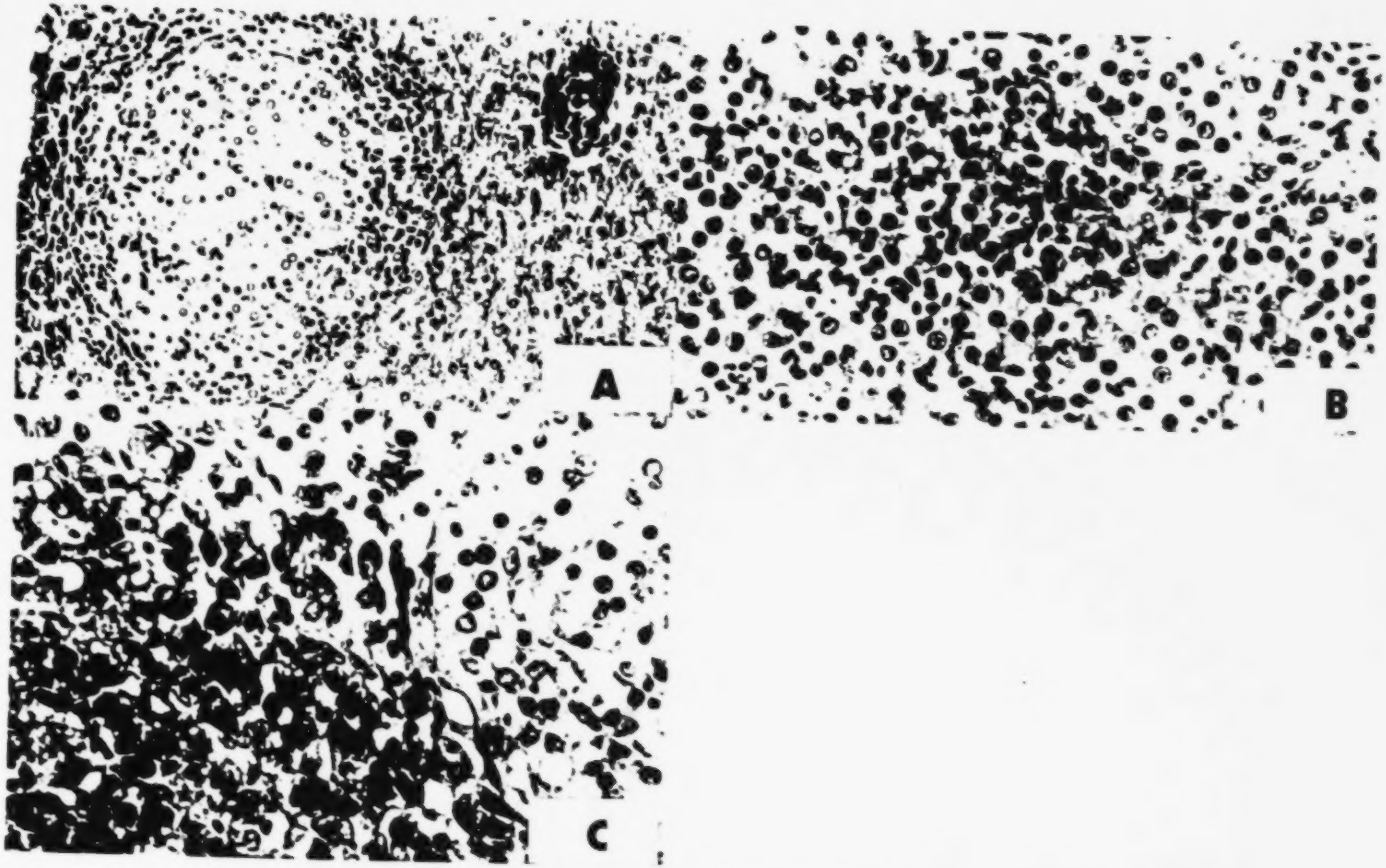

Figure 46. Alterations in Livers of Fundulus Heteroclitus.

A. Higher magnification of liver granuloma. Round cell infiltrate is shown at periphery. FBPA 4 months. H&E X 200. B. Accumulation of acidophilic material in lining cells of fundulus liver after FBPA. H&E X 200. C. Fundulus liver after 4 months FBPA exposure. Note basophilic focus near granulomas. H&E X 400.

SECTION VII

REFERENCES

1. WHO. Prevention of Cancer Technical Report. Series 276, Geneva, 1964.

2. Boyland, E. The correlation of experimental carcinogenesis and cancer in man. Prog. Exp. Tumor Res. 11:222-234, 1969.

3. Epstein, S.S. Control of chemical pollutants. Nature, 228:816-819, 1970.

4. Revelle, R. The Ocean. Scientif. Amer. 221:55-65, 1968.

5. Kurland, L.T., Faro, S.N., and Siedler, H. Minamata disease: The outbreak of neurological disorder in Minamata, Japan and its relationship to imported seafood contaminated by mercury compounds. World Neurol. 1:370-395, 1960.

6. Garrison, A.W. Analysis of organic compounds in water to support health effects studies. Annals N.Y. Acad. Sci. 298:2-19, 1977.

7. Keith, L.H., Garrison, A.W., Allen, F.R., Carter, M.H., Floyd, T.L., Pope, J.D., and Thurston, A.D. Identification of organic compounds in drinking water from thirteen U.S. cities. In: Identification and analysis of organic pollutants in water, L.H. Keith, ed. Chapter 22, Ann Arbor Science Publishers, Inc., Ann Arbor, Michigan, 1976.

8 Kraybill, H.E. Introductory remarks: Overview on aquatic pollutants and their biologic effects. Annals N.Y. Acad. Sci. 298:1, 1977.

9. Wellings, S.R. Neoplasia and primitive vertebrate phylogeny: echinoderms, prevertebrates and fishes - A review. Nat. Cancer Inst. Mongr. 31:59-121, 1969.

10 Mawdesley-Thomas, L.E. Neoplasia in fish. In: The Pathology of Fishes, W.E. Ribelin and G. Migaki, eds. University of Wisconsin Press, Madison. pp. 805-870, 1975.

11. Harshbarger, J.C. Role of the registry of tumors in lower animals in the study of environmental carcinogenesis in aquatic animals. Annals N.Y. Acad. Sci. 298:280-289, 1977.

12. Stewart, H.L. Discussion paper: Enigmas of cancer in relation to neolasms of aquatic animals. Annals N.Y. Acad. Sci. 298:305-315, 1977.

13. Matsushima, T., and Sugimura, T. Experimental carcinogenesis in small aquarium fishes. Prog. Exp. Tumor Res. 20: 367-379, 1976.

14. Pliss, G.B., and Khudoley, V.V. Tumor induction by carcinogenic agents in aquarium fish. J. Nat. Cancer Inst. 55:129-136, 1975.

15. Sato, S., Matsushima, T., Tanaka, N., Sugimura, T., and Takashima, F. Hepatic tumors in the guppy (*Lebistes reticulatas*) induced by aflatoxin B , dimethylnitrosamine, and 2-acetylaminofluorene. J. Nat. Cancer Inst. 50:767-778, 1973.

16. Ashley, L.M. Pathology of fish fed aflatoxins and other anti-metabolites. *In*: A Symposium on Diseases of Fishes and Shell Fishes, S.F. Snieszko, ed. American Fisheries Society, Washington, D.C., 1970, pp. 366-379.

17. Sinnhuber, R.O., Hendricks, J.D., Wales, J.H., and Putnam, G.B. Neoplasms in rainbow trout, a sensitive animal model for environmental carcinogenesis. Annals N.Y. Acad. Sci. 298:398-408, 1977.

18. Wales, J.H., and Sinnhuber, R.O. Hepatomas induced by aflatoxin in the sockeye salmon (*Oncorhynchus nerka*). J. Nat. Cancer Inst. 48: 1529-1530, 1972.

19. Wolf, H., and Jackson, E.W. Hepatomas in rainbow trout: Descriptive and experimental epidemiology. Science 142:676-678, 1963.

20. Stanton, M.F. Hepatic neoplasms of aquarium fish exposed to *Cycas circinalis* Fed. Proc. 25:661, 1966.

21. Aoki, K., and Matsudaira, H. Induction of hepatic tumors in a teleost (*Oryzias latipes*) after treatment with methylazoxymethanol acetate: Brief communication. J. Nat. Cancer Inst. 59:1747-1749, 1977.

22. Stanton, M.F. Diethylnitrosamine-induced hepatic degeneration and neoplasia in the aquarium fish, *Brachydanio rerio*. J. Nat. Cancer Inst. 34:117-130, 1965.

23. Ishikawa, T., Shimamine, T., and Takayama, S. Histologic and electron microscopy observations on diethylnitrosamine-induced hepatomas in small aquarium fish (*Oryzias latipes*). J. Nat. Cancer Inst. 55: 909-916, 1975.

24. Ermer, M. Versuche mit cancerogenen mitteln bei kurzlebigen fischarten. Zool. Anz. 184:175-193, 1970.

25 Stich, H.F., Acton, A.B., Dunn, B.P., Oishi, K., Yamazaki, F., Harada, T., Peters, G., and Peters, N. Geographic variations in tumor prevalence among marine fish populations. Int. J. Cancer 20: 780-791, 1977.

26. Stich, H.F., Acton, A.B., Oishi, K., Yamazaki, F., Harada, T., Hibino, T., and Moser, H.G. Systematic collaborative studies on neoplasms in marine animals as related to the environment. Annals N.Y. Acad. Sci. 298:374-388, 1977.

27. Stich, H.F., and Acton, A.B. The possible use of fish tumors in monitoring for carcinogens in the marine environment. Prog. Exp. Tumor Res. 20:44-54, 1976.

28 Miller, E.C., and Miller, J.A. Biochemical mechanisms of chemical carcinogenesis. In: Molecular Biology of Cancer, H. Busch, ed. Academic Press, New York, 1974. pp. 377-402.

29. Miller, J.A. Carcinogenesis by chemicals--an overview. G.H.A. Clowes Memorial Lecture. Cancer Res. 30:559-576, 1970.

30 Dallner, G. Studies on the structural and enzyme organization of the membranous elements of liver microsomes. Acta. Pathol. Microbiol. Scand., Suppl. 166, 1, 1963.

31. Gillette, J.R., Davis, D.C., and Sasame, H.A. Cytochrome P-450 and its role in drug metabolism. Annu. Rev. Pharmacol. 12:57-84, 1972.

32. Ullrich, V. The mechanism of cytochrome P-450 action. In: Microsomes and Drug Oxidations, V. Ullrich, A. Hildebrandt, I. Roots, R. Estabrook, and A. Conney, eds. Pergamon Press, New York, 1977. pp. 192-201.

33. Lu, A.Y.H. Liver microsomal drug-metabolizing enzyme system: Functional components and their properties. Fed. Proc. 35:3460-3463, 1976.

34. Yang, C.S. The organization and interaction of monooxygenase enzymes in the microsomal membrane. Life Sci. 21:1047-1058, 1977.

35 De Pierre, J.W., and Dallner, G. Structural aspects of the membrane of the endoplasmic reticulum. Biochim. Biophys. Acta 415:411-471, 1975.

36. Orrenius, S., and Ernster, L. Enzymatic properties of membrane associated cell fractions as related to drug metabolism. In: Cell Membranes--Biological and Pathological Aspects, G. Richter and D. Scarpelli, eds. Williams and Wilkins, Baltimore, 1971. pp. 38-53.

37 Gigon, P.L., Gram, T.E., and Gillette, J.R. Studies on the rate of reduction of hepatic microsomal cytochrome P-450 by reduced nicotinamide adenine dinucleotide phosphate. Effect of drug substrates. Mol. Pharmacol. 5:109-122, 1969.

38. Mannering, G.J. Properties of cytochrome P-450 as affected by environmental factors: Qualitative changes due to administration of polycyclic hydrocarbons. Metabolism 20:228-245, 1971.

39. Gillette, J.R. Factors that affect the stimulation of the microsomal drug enzymes induced by foreign compounds. Advances in Enzyme Regulation 1:215-223, 1963a.

40. Gillette, J.R. Metabolism of drugs and other foreign compounds by enzymatic mechanisms. In: Progress in Drug Research, E. Jucker ed. Vol. 6, Birkhauser Verlag, Basel, 1963b. pp. 13-73.

41 Fouts, J.R. Liver smooth endoplasmic reticulum "microsomal" drug-metabolizing enzyme system. Methods in Pharmacol. 1:287-325, 1971.

42. Conney, A.H. Pharmacological implications of microsomal enzyme induction. Pharmacol. Rev. 19:317-366, 1967.

43. Hinton, D.E., Glaumann, H., and Trump, B.F. Studies on the cellular toxicity of polychlorinated biphenyls (PCBs). I. Effect of PCBs on microsomal enzymes and on synthesis and turnover of microsomal and cytoplasmic lipids of rat liver - a morphological and biochemical study. Virchows Archiv. 27:279-306, 1978.

44. Albert, R.E. Skin carcinogenesis. In: Cancer of the Skin: Biology, Diagnosis, Management, R. Andrade, S.L. Gumport, Popkin, G.L., and T.D. Rees, eds. W.B. Saunders Co., Philadelphia, 1976. pp. 111-136.

45 Saffiotti, U., Cefis, F., and Kolb, L. A method for the experimental induction of bronchogenic carcinoma. Cancer Res. 28:104-124, 1968.

46. Heidelberger, C. Chemical carcinogenesis. Ann. Rev. Biochem. 44: 79-121, 1975.

47 Grover, P.L., and Sims, P. Enzyme catalyzed reactions of polycyclic hydrocarbons. Biochem. J. 110:159-160, 1968.

48 Gelboin, H.V. A microsome-dependent binding of benzo(a)pyrene to DNA. Cancer Res. 29:1272-1276, 1969.

49. Slekirk, J.K. Benzo(a)pyrene carcinogenesis: A biochemical selection mechanism. J. of Toxicol. and Environ. Health 2:1245-1258, 1977.

50. DePierre, J.W., and Ernster, L. The metabolism of polycyclic hydrocarbons and its relationship to cancer. Biochim. et Biophys. Acta 4:149-176, 1978.

51. Selkirk, J.K., Croy, R.G., and Gelboin, H.V. Benzo(a)pyrene metabolites: Efficient and rapid separation by high pressure liquid chromatography. Science 184:169-171, 1974a.

52. Yang, S.K., Selkirk, J.K., Plotkin, E.V., and Gelboin, H.V. Kinetic analysis of the metabolism of benzo(a)pyrene to phenols, dihydrodiols, and quinones by high pressure liquid chromatography compared to analysis by AHH assay, and the effect of enzyme induction. Cancer Res. 35:3642-3650, 1975.

53. Heidelberger, C. Chemical carcinogenesis. Cancer 40:430-433, 1977.

54. Harris, C.C., Valerio, G.M., Frank, A.L., Kaufman, D.G., Barrett, L.A., McDowell, E.M., and Trump, B.F. Carcinogeneic polynuclear hydrocarbons bind to macromolecules in cultured human bronchi. Nature 252:68-69, 1974.

55 Autrup, H., Harris, C.C., Stoner, G.D., Jesudason, M.L., and Trump, B.F. Binding of chemical carcinogens to macromolecules in cultured human colon. J. Natl. Cancer Inst. 59:351-354, 1977.

56. Ames, B.N., Durston, W.E., Yamasaki, E., and Lee, F.D. Carcinogens are mutagens: A single test system combining liver homogenate for activation and bacteria for detection. Proc. Nat. Acad. Sci. 70: 2281-2285, 1973.

57. McCann, J., Choi, E., Yamasaki, E., and Ames, B.N. Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals. Proc. Nat. Acad. Sci. 72:5135-5139, 1975.

58. McCann, J.B., and Ames, B.N. Detection of carcinogens as mutagens in Salmonella/microsome test: Assay of 300 chemicals. Discussion. Proc. Nat. Acad. Sci. 73:950-954, 1976.

59. Weisburger, J.H. and Williams, G.M. Carcinogen Testing: Current Problems and New Approaches. Science 214:401-407, 1981.

60. Brodie, B.B. and Maickel, R.P. Comparative biochemistry of drug metabolism. Proc. First International Pharmacology Meeting. 6: 299-324, 1962.

61. Adamson, R.H. Drug metabolism in marine vertebrates. Fed. Proc. 26:1047-1055, 1967.

62. Inano, H., Mari, K., Tamaoki, B., and Gustafsson, J.A. *In vitro* metabolism of testosterone in hepatic tissue of a hagfish, *Eptatretus burgeri*. Gen. Comp. Endo. 30:258-266, 1976.

63. Buhler, D.R., and Rasmussen, M. The oxidation of drugs by fishes. Comp. Biochem. Physiol. 25:223-239, 1968.

64. Pohl, R.J., Bend, J.R., Guarino, A.M., and Fouts, J.R. Hepatic microsomal mixed-function oxidase activity of several marine species from coastal Maine. Drug Metabolism and Disposition 2:545-555, 1974.

65. Stanton, R.H., and Kahn, M.A. Mixed-function oxidase activity toward cyclodiene insecticides in bass and bluegill sunfish. Pestic. Biochem. Physiol. 3:351-357, 1973.

66. Ahokas, J.T., Pelkonen, O., and Karki, N. Cytochrome P-450 and drug induced spectral interactions in hepatic microsomes of trout, *Salmo trutta lacustris*. Acta Pharmacol. Toxicol. 38:440-449, 1976.

67. Bend, J.R., Pohl, R.J., Arine, E., and Philpot, R.M. Hepatic microsomal and solubilized mixed-function oxidase systems from the little skate, Raja *erinacea*, a marine elasmobranch. *In*: Microsomes and Drug Oxidations, V. Ullrich, A Hildebrandt, I. Roots, R. Estabrook, and A. Conney, eds. Pergamon Press, New York, 1977a. pp. 16-169.

68. Pedersen, M.G., and Hershberger, W.K. Metabolism of 3,4-benzpyrene in rainbow trout (*Salmo gairdneri*). Bull. of Environ. Contam. Tox. 12 (4):481-486, 1974.

69. Bend, J.R., James, M.O., and Dansette, P.P. *In vitro* metabolism of xenobiotics in some marine animals. Ann. N.Y. Acad. Sci. 298:505-521, 1977b.

70. Gruger, E.H., Wekell, M.M., Numotot, P.T., and Craddock, D.R. Induction of hepatic aryl hydrocarbon hydroxylase in salmon exposed to petroleum and polychlorinated biphenyls, separate, and together, in food. Bull. Environ. Contam. Toxicol. 17:517-520, 1977.

71. James, M.O., Khan, M.A.Q., and Bend, J.R. Hepatic microsomal mixed function oxidase activities in several marine species common to coastal Florida. Comp. Biochem. Physiol. 62C:155-164, 1979.

72. Pedersen, M.G., Hershberger, W.K., Zachariah, P.K., and Juchau, M.R. Hepatic biotransformation of environmental xenobiotics in six strains of rainbow trout (*Salmo gairdneri*). J. Fish. Res. Bd. Can. 33:666-675, 1976.

73. Stegeman, J.J. Influence of environmental contamination on cytochrome P-450 mixed-function oxygenases in fish: Implication for recovery in the Wild Harbor Marsh. J. Fish. Res. Bd. Can. 35:668-674, 1978.

74. Stegeman, J.J., and Chevion, M. Sex differences in cytochrome P-450 and mixed function oxygenase activity in gonadally mature trout. Biochem. Pharmacol. 29'553-558, 1980.

75. Payne, J.F. Field evaluaton of BP hydroxylase induction as a monitor for marine petroleum pollution. Science 191:945-946, 1976.

76. Gerhart, E.H., and Carlson, R.M. Hepatic mixed function oxidase activity in rainbow trout exposed to several polycyclic aromatic compounds. Environ. Res. 17:284-295, 1978.

77. Elcombe, C.R., and Lech, J.J. Induction of monooxygenation in rainbow trout by polybrominated biphenyls: A comparative study. Environ. Health Perspectives 23:309-314, 1978.

78. Lidman, U., Forlin, L., Molander, D., and Exelson, G. Induction of drug metabolizing system in rainbow trout (Salmo gairdneri) liver by polychlorinated biphenyls (PCBs). Acta Pharmacol. Toxicol. 39:262-272, 1976.

79. Lipsky, M.M., Klaunig, J.E., and Hinton, D.E. Comparison of acute response to polychlorinated biphenyl in liver of rat and channel catfish: A biochemical and morphological study. J. Toxicol. Environ. Health. 4:107-121, 1978.

80. Klaunig, J.E., Lipsky, M.M., Trump, B.F., and Hinton, D.E. Biochemical and ultrastructural changes in teleost liver following subacute exposure to PCB. J. Environ. Path. and Toxicol. 2:953-963, 1979.

81. Hill, D.W., Hejtmancik, E., and Camp, B.J. Induction of hepatic microsomal enzymes by Aroclor 1254 in Ictalurus punctatus (channel catfish). Bull. Environ. Contam. Toxicol. 16:495-502, 1976.

82 Statham C.N., Elcombe, C.R., Szyjka, S.P., and Lech, J.J. Effect of polycyclic aromatic hydrocarbons on hepatic microsomal enzymes and disposition of methylnaphthalene in rainbow trout in vivo. Xenobiotica. 8:67-71, 1978.

83. Ahokas, J.T., Pelkonen, O., and Karki, N.J. Metabolism of polycyclic hydrocarbons by a hightly active aryl hydrocarbon hydroxylase system in the liver of the trout species. Biochem. and Biophys. Commun. 63(3):635-641, 1975.

84. DeWaide, J.H. Alterations in microsomal drug oxidation in thermally acclimated fish. Arch. Int. Pharmacodyn. Ther. 189:377-379, 1971.

85 Stanton, R.H., and Khan, M.A. Components of the mixed function oxidase system of hepatic microsomes of freshwater fish. Gen. Pharmacol. 6:284-294, 1975.

86. Hogan, J.W., and Knowles, C.O. Metabolism of Diazon by fish liver microsomes. Bull. Environ. Contam. Toxicol. 8:61-64, 1972.

87. Lotlikar, P.D., Miller, E.C., Miller, J.A., and Halver, H.E. Metabolism of the carcinogen 2-acetylaminoflourene by rainbow trout. Proc. Soc. Exper. Biol. Med. 141:765-768, 1967.

88 Diamond, L., and Clark, H.F. Comparative studies on the interaction of benzo(a)pyrene with cells derived from poikilothermic and hemeothermic vertebrates I. Metabolism of benzo(a)pyrene. J. Nat. Cancer Inst. 45:1005-1011, 1970.

89 Stephan, C.E. Methods for acute toxicity tests with fish, macroinvertebrates, and amphibians by the committee on methods for toxicity tests with aquatic organisms. National Environmental Research Center Office of Research and Development. EPA-660/3-75-009, April 1975.

90. McFarland, W.N., and Klontz, G.W. Anesthesia in fishes. Fed. Proc. 28:1535-1540, 1969.

91. Hinton, D.E. Perfusion fixation of whole fish for electron microscopy. J. Fish. Res. Bd. Can. 32:416-422, 1975.

92 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. Protein measurements with the folin phenol reagent. J. Biol. Chem. 193:265-275, 1951.

93. Omura, T., and Sato, R. The carbon monoxide binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. II. Solubilization, purification, and properties. J. Biol. Chem. 239: 2370-2385, 1964.

94 Gnosspelius, Y., Thor, H., and Orrenius, S. A comparative study on the effects of phenobarbital and 3,4-benzpyrene on the hydroxylating enzyme system of rat liver microsomes. Chem. Biol. Interact. 1:125-137, 1969.

95. Nash, J. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. Biochem. J. 55:416-421, 1953.

96. Dallner, G., Siekevitz, P., and Palade, G.E. Biogenesis of endoplasmic reticulum membranes. I. Structural and chemical differentiation in developing rat hepatocyte. J. Cell Biol. 30:73-96, 1966.

97. Nebert, D.W., and Gelboin, H.V. Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. J. Biol. Chem. 243:6242-6249, 1968.

98. McDowell, E.M., and Trump, B.F. Histologic fixatives suitable for diagnostic light and electron microscopy. Arch. Pathol. Lab. Med. 100:405-414, 1976.

99. Luft, J.H. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9:409-414, 1961.

100 Trump, B.F., Smuckler, E.A., and Benditt, E.P. A method of staining epoxy sections for light microscopy. J. Ultrastruct. Res. 5:343-348, 1961.

101. Porter, K.R., Kelley, D., and Andrews, P.M. The preparation of cultured cells and soft tissues for scanning electron microscopy. Proceedings of the fifth annual stereoscan colloquim. Kent Cambridge Scientific Co., Morton Grove, Illinois, 1972. pp. 1-19.

102. Barrett, L.A., McDowell, E.M., Frank, A.L., Harris, C.C., and Trump, B.F. Long-term organ culture of human bronchial epithelium. Cancer Res. 36:1003-1010, 1976.

103. Ames, B.N., McCann, J., and Yamasaki, E. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsomes mutagenicity test. Mutation Research 31:347-364, 1975.

104. Huberman, E., and Sachs, L. DNA binding and its relationship to carcinogenesis by different polycyclic hydrocarbons. Int. J. Cancer 19:122-127, 1977.

105. Kraybill, H.F. Distribution of chemical carcinogens in aquatic environments. Prog. Exp. Tumor Res. 20:3-34, 1976.

106. Morris, H.P., Velat, C.A., and Wagner, D.R. Carcinogenicity of some ingested acetylated mono- and diaminobiphenyl compounds in the rat. J. Nat. Cancer Inst. 18:101-115, 1957.

107. Dees J., Reuber, M. and Trump, B.F. Studies on adenocarcinoma of the kidney. I. Ultrastructure of rat renal adenocarcinoma induced by N-(4-fluoro-4-biphenyl)acetamide. J. Nat. Cancer Inst. 57:779-794, 1976.

108. Heatfield, B.M., Hinton, D.E., and Trump, B.F. Studies on adenocarcinoma of the kidney. II. Enzyme histochemistry of renal adenocarcinoma induced in rats by N-(4'-fluoro-4-biphenylyl)acetamide. J. Nat. Cancer Inst. 57:795-808, 1976.

109. Hinton, D.E., Lipsky, M.M., Heatfield, B.M. and Trump, B.F. Opposite effects of the environmental pollutant Pb upon N-4-4'-fluorobiphenylyl acetamide-induced liver and kidney carcinogenesis. Bull. Environ. Contam. Toxicol. 23:464-469, 1979.

110. Hinton, D.E., Heatfield, B.M., Lipsky, M.M., and Trump, B.F. Chemically induced renal tubular carcinoma. Amer. J. Pathol. 100: 317-320, 1980.

111. Scarpelli, D.G. Drug metabolism and aflatoxin-induced hepatoma in rainbow trout (Salmo gairdneri). Prog. Exp. Tumor Res. 20:339-350, 1976.

112. Scarpelli, D.G. General Discussion to Part IV. Biological Effects on Marine Animals: Health Implication for Man. Aquatic Pollutants and Biologic Effects with Emphasis on Neoplasia, H.F. Kraybill, C.J. Dawe, J.C. Harshbarger, and R.G. Tardiff, eds. Ann. N.Y. Acad. Sci. 298:463-481, 1977.

113. Couch, J.A. Histopathological effects of pesticides and related chemicals on the livers of fishes. In: Pathology of Fishes, W.E. Ribelin and G. Migaki, eds. University of Wisconsin Press, Madison, 1975. pp. 559-584.

114. Hinton, D.E., and Pool, C.R. Ultrastructure of the liver in channel catfish, Ictalurus punctatus (Rafinesque). J. Fish. Biol. 8:209-220, 1976.

115. Scarpelli, D.G., Greider, M.H., and Frajola, W.J. Observations of hepatic cell hyperplasia, adenoma, and hepatoma in rainbow trout (Salmo gairdneri). Cancer Res. 23:848-857, 1963.

116. Weis, P. Ultrastructural changes induced by low concentrations of DDT in the livers of the zebrafish and the guppy. Chem. Biol. Interactions 8:25-30, 1974.

117. Smuckler, E.A., and Arcasoy, M. Structural and functional changes of endoplasmic reticulum of hepatic parenchymal cells. Int. Rev. Exp. Path. 7:305-418, 1969.

118. Stegeman, J.J., and Binder, R. High benzo(a)pyrene hydroxylase activity in the marine teleost fish Stenotomus versicolor. Biochem. Pharmacol. 28:1686-1688, 1979.

119. Weibel, F.J., Leutz, J.D., Diamond, L., Gelboin, H.V. Aryl hydrocarbon (benzo(a)pyrene) hydroxylase in microsomes from rat tissues: differential inhibition and stimulation by benzoflavones and organic solvents. Arch. Biochem. Biophys. 144:78-86, 1971.

120 Diamond, L., and Gelboin, H.V. Alpha-napthoflavone: An inhibitor of hydrocarbon cytotoxicity and microsomal hydroxylase. Science 166:1023-1025, 1969.

121. Gelboin, H.V., Weibel, F.J., and Diamond, L. Dimethylbenzanthracene tumorigenesis and aryl hydrocarbon hydroxylase in mouse skin: inhibition by 7,8-benzoflavone. Science 170:169-171, 1970.

122. Philpot, R.M. and Bend, J.R. Benzpyrene hydroxylase activity in hepatic microsomal and solubilized systems containing rabbit or rat cytochrome P-448 or P-450. Life Sci. 16:985-998, 1975.

123 Dewaide, J.H., and Henderson, P.Th. Seasonal variation of hepatic drug metabolism in the roach, Leuciscus rutilus L. Comp. Biochem. Physiol. 32:489-497, 1970.

124. Hoar, W.S. Endocrine organs. In: The Physiology of Fishes, M.E. Brown, ed. Academic Press, New York, 1957. pp. 245-321.

125 Kato, R. Sex-related differences in drug metabolism. Drug Metab. Rev. 3:1-31, 1974.

126. Alvares, A.P., Schilling, G., Levin, W., Kuntzman, R. Studies on the induction of CO-binding pigments in liver microsomes by phenobarbital and 3-methylcholanthrene. Biochem. Biophys. Res. Commun. 29:521-526, 1967.

127 Pezzuto, J.M., Yang, C.S., Yang, S.K., McCourt, D.W., and Gelboin, H.V. Metabolism of benzo(a)pyrene and (-)-trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene by rat liver nuclei and microsomes. Cancer Res. 38:1241-1245, 1978.

128. Ahokas, J.T., Saarni, H., Nebert, D.W., and Pelkonen, O. The in vitro metabolism and covalent binding of benzo(a)pyrene to DNA catalysed by trout liver microsomes. Chem. Biol. Interact. 25:103-111, 1979.

129. Selkirk, J.K., Yang, S.K., and Gelboin, H.V. Analysis of benzo(a)pyrene in rat liver microsomes. In: Chemistry, Metabolism and Carcinogenesis, R.I. Freudenthal and P.W. Jones, eds. Raven Press, New York, 1976. pp. 153-169.

130. Selkirk, J.K., Croy, R.G., Roller, P.P., and Gelboin, H.V. High pressure liquid chromatographic analysis of benzo(a)pyrene metabolism and covalent binding and the mechanism of action of 7,8-benzoflavone and 1,2-epoxy-3, 3,3-trichloropropane. Cancer Res. 34:3437-3480 1974b.

131 Yang, S.K., Gelboin, H.V., Trump, B.F., Autrup, H., and Harris, C.C. Metabolic activation of benzo(a)pyrene and binding to DNA in cultured human bronchus. Cancer Res. 37:1210-1215, 1977.

132. Wood A.W., Wislocki, P.G., Chang, R.L., Levin, W., Lu, A.Y.H., Yagi, H., Hernandex, O., Jerina, D.M., and Conney, A.H. Mutagenicity and cytotoxicity of benzo(a)pyrene benzo-ring epoxides. Cancer Res. 36:3358-3366, 1976.

133 Sims, P., and Grover, P.L. Epoxides in polycyclic aromatic hydrocarbon metabolism and carcinogenesis. Adv. Cancer Res. 20:165-274, 1974.

134. Daudel, P., Duquesne, M. Vigney, P., Grover, P.L., and Sims, P. Fluorescence spectral evidence that benzo(a)pyrene-DNA products in mouse skin arise from diol-epoxides. Fed. Europ. Biochem. Soc. Letters 57:250-253, 1975.

135. Ahokas, J.T., Paakkonen, R., Ronnholm, K., Raunio, V., Karki, N., and Pelkonen, O. Oxidative metabolism of carcinogenesis by trout liver resulting in protein binding and mutagenicity. In: Microsomes and Drug Oxidations, V. Ullrich, A. Hildebrandt, I. Roots, R. Estabrook, and A. Conney, eds. Pergamon Press, New York, 1977. pp. 435-441.

136 Stott, W.T., and Sinnhuber, R.O. Trout hepatic enzyme activation of aflatoxin B, in a mutagen assay system and the inhibitory effects of PCBs. Bull. Environ. Contam. Toxicol. 19:35-41, 1978.

137. Thorgiersson, S.S., and Nebert, D.W. The Ah locus and the metabolism of chemical carcinogens and other foreign compounds. Adv. Cancer Res. 25:149-193, 1977.

138 Fry, J R., and Brigdes, J.W. Use of primary hepatocyte cultures in biochemical toxicology. In: Reviews in Biochemical Toxicology, E. Hodgson, J.R. Bend, and R.M. Philpot, eds. Elsevier North Holland, Inc , New York, 1979.

139 Green, M.H.L., Bridges, B.A., Rogers, A.M., Horspool, G., Murial, W.J., Bridges, J.W., and Fry, J.R. Mutagen screening by a simplified bacterial fluctuation test: Use of microsomal preparations and whole liver cells for metabolic activation. Mutation Res. 48:287-294, 1977.

140 Yang S.K., Deutsch, J., and Gelboin, H.V. Benzo(a)pyrene Hydrocarbons and Cancer, Vol. 1, H.V. Gelboin, and P.O. TS'O, eds. Academic Press, New York, 1978. pp. 205-229.

141. Williams, G.M. The use of liver epithelial cell cultures for the study of chemical carcinogenesis. Am J. Path. 85:739-751, 1976.

142. Williams, G M. Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell cultures. Cancer Res. 37:1845-1851, 1977.

143 U.S. Bureau of Sport Fisheries and Wildlife. Report to the Fish Farmers. Resource Publication 83. Washington, D.C., 1970. pp. 1-124.

144 Guerin, M.R. Energy sources of polycyclic aromatic hydrocarbons. In: Polycyclic Aromatic Hydrocarbons and Cancer, Vol. 1, H.V. Gelboin and P.O. TS'O, eds. Academic Press, New York, 1978. pp. 3-42.

145. Schoor, W.P., and Couch, J.A. Correlation of mixed-function oxidase activity with ultrastructural changes in the liver of a marine fish. Cancer Biochem. Biophys. 4:95-103, 1979.